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TITLE: Characterization of a SUMO Ligase that is Essential for DNA Damage-Induced NF-Kappa B Activation

PRINCIPAL INVESTIGATOR: Angela M. Mabb
Shigeki Miyamoto, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin-Madison
Madison, WI 53706

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Angela M. Mabb
Shigeki Miyamoto, Ph.D.

Email: ammabb@wisc.edu

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14. ABSTRACT It has been recently proposed that inhibition of NF-?B may be a therapeutic target for the treatment of ER- breast cancers. As of now, the majority of NF-?B inhibitors focus on the key signal integrating complex known as the I?B kinase (IKK) complex. Since NF-?B plays a major role in many essential physiological processes in the cell, global inhibition of NF-?B at the central IKK complex could allow for increased risk of side effects as well as the desirable effects on cancer cell death. Hence, identification of specific, novel molecular targets in the NF-?B signaling pathway may lead to the identification of more specific NF-?B inhibitors. Our hypothesis is that PIASy, a SUMO ligase, is essential for DNA damage induced NF-?B activation, however is not critical for classical activation of NF-?B, leaving the more physiological pathway intact. We reveal that PIASy is signaling at the level of NEMO SUMOylation, a posttranslational modification that we recently identified being critical for DNA damage induced activation of NF-?B. Reduction of PIASy through siRNA caused inhibition of NF-?B in response to multiple DNA damaging agents commonly used in anti-cancer therapy. We provide strong evidence that PIASy is working at the level of NEMO SUMOylation and propose that PIASy is the SUMO ligase for NEMO. Furthermore, we show that the catalytic activity of PIASy is essential for NF-?B activation and hence suggest that inhibition of PIASy may be used as a more specific inhibitor in anti-cancer therapy to treat ER- breast cancer.

15. SUBJECT TERMS
PIAS•/PIASy, NEMO, NF-•B, SUMO

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Annual Summary Report

I. Introduction

Background:

Nuclear Factor-κB (NF-κB) is a transcription factor that regulates a diverse subset of genes involved in immune function, growth control, development, and regulation of apoptosis. NF-κB exists in the cell in inactive cytoplasmic complexes, with the predominant complex being p65/p50 dimers. NF-κB can be activated by a wide variety of stimuli at the cell surface such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and lipopolysaccharide (LPS). NF-κB can also be activated in response to various DNA damaging agents, such as ionizing radiation and Topoisomerase II inhibitors (e.g. etoposide and doxorubicin/adriamycin). Activation of NF-κB in response to various DNA damaging agents is less rapid than extracellular stimuli and generally results in the activation of anti-apoptotic genes. The early key molecular events involved in NF-κB activation with various stimuli are diverse; however a key component in this signaling pathway with most stimuli is the IKK complex that is composed of three major proteins in the cytoplasm of the cell. One protein, NEMO serves as an adaptor/scaffolding component which is essential for IKK complex activity to ultimately activate NF-κB. NF-κB activation has also been suggested to play a role in resistance to therapy in advanced breast cancers. Importantly, estrogen receptor negative (ER-) breast cancers tend to have high constitutive levels of active NF-κB and activate NF-κB in response to DNA damaging anticancer agents. It has been recently proposed that inhibition of NF-κB may be a potential therapeutic target for ER- breast cancers. As of now, the majority of NF-κB inhibitors focus inhibiting the IKK complex. One potential issue with inhibiting the IKK complex is that NF-κB mediates a variety of physiologically vital processes besides those involved in the regulation of apoptosis. As a result, nonspecific inhibition of NF-κB's various target genes could allow for an increased risk of side effects and unpredictable effects on apoptosis of breast cancer cells. One solution to this dilemma is inhibition of specific NF-κB pathways upstream of IKK activation in response to DNA damaging agents. If one could selectively inhibit the NF-κB signaling pathways induced by DNA damaging anticancer agents and leave other important NF-κB signaling pathways (such as TNFα and IL-1 signaling) intact, then enhanced cancer cell death may be achieved with fewer side effects

Objective/Hypothesis: PIASγ/PIASy is the SUMO ligase for NEMO and inhibition of this specific ligase may provide a novel target for chemotherapeutic treatment regimens against ER- breast cancer.

II. Body

Aim1: To understand the mechanisms governing NEMO SUMOylation

In this grant, we proposed if PIASy was directly affecting NEMO SUMOylation, then it should be able to enhance NEMO SUMOylation *in vitro*. We initially showed that *in vitro* translated NEMO in rabbit reticulocyte extracts could be SUMO modified by immunopurified PIASy. However, we were unable to show that GST-NEMO could be SUMOylated *in vitro* and were unsuccessful in purifying His-PIASy with high purity and quantity. We were able to obtain

highly purified recombinant His-PIASy in collaboration with Dr. Yoshiaka Azuma. We utilized this His-PIASy in an *in vitro* SUMOylation assay with purified recombinant His-NEMO that we generated in the lab (Figure 1). We found that PIASy can promote NEMO SUMOylation in a complete recombinant system and this could be reversed by the catalytic domain of a SUMO protease, Senp1.

Figure 1:

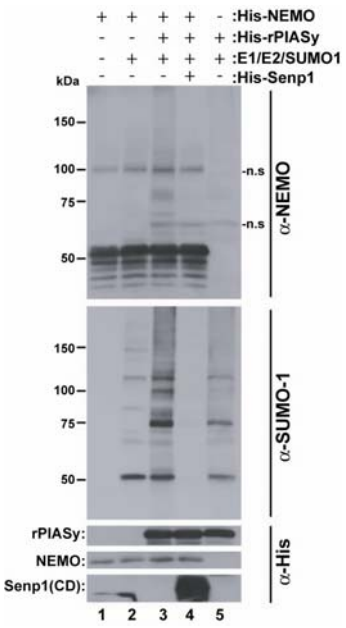

Figure 1: *In vitro* SUMOylation assay was performed using 0.75 μg of recombinant His-NEMO and 1 μg of recombinant His-rPIASy at 30°C for 75 min. His-SENP1 was added after SUMOylation in lane 4. Samples were terminated in 2xSDS sample buffer, run on an SDS-PAGE gel and blotted with anti-NEMO (top), anti-SUMO-1 (middle) and anti-His antibodies (bottom three blots)

We have previously shown that PIASy can inducibly interact with endogenous NEMO. However, we would like to examine and isolate an endogenous NEMO:PIASy complex to identify novel binding partners and understand how this complex is functioning in the cell. Since a suitable commercial antibody is not available, we decided to generate our own for our future studies with PIASy. In collaboration with Neoclone, (a Madison based company), we provided them with purified recombinant His-PIASy. Ascites with cross reactivity for His-PIASy was given to us to test for reactivity against PIASy. We currently have ascites that tests positive against human PIASy that will be suitable for our future studies. Furthermore, we have identified RSK1, a protein recently shown to be required for DNA damage induced NF-κB activation as a binding partner for PIASy (Figure 2).

Figure 2:

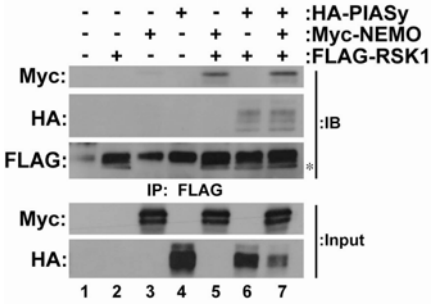

Figure 2: HEK293 cells were transfected with FLAG-RSK1, Myc-NEMO, and HA-PIASy. Cells were lysed and immunoprecipitated using anti-FLAG antibody. Western blotting was performed using anti-Myc, anti-HA, and anti-FLAG antibodies.

We are currently trying to understand the role of RSK1 kinase in the PIASy:NEMO complex and how it signals in the DNA damage induced NF-κB activation pathway.

Aim2: Determine if PIASγ/y dictates the ability of breast cancer cells to activate NF-κB basally and in response to DNA damaging agents.

We initially proposed that PIASy expression levels may dictate the ability of breast cancer cells to activate NF-κB and have high constitutive NF-κB activity. For instance, we have found that the MDA-MB-231(ER-) breast cancer cell line efficiently activates NF-κB in response to DNA damaging agents. Interestingly, MDA-MB-231 breast cancer cell lines have high constitutive NF-κB activity and this activity is increased upon stimulation with DNA damaging agents. In contrast, MCF-7 (ER+) breast epithelial cell lines are extremely inefficient to activate NF-κB in response to DNA damaging agents, even though these cells are capable of activating NF-κB when stimulated with the non-DNA damaging agent TNFα. We proposed that PIASy protein levels may also dictate whether or not cell lines can activate NF-κB in response to DNA damaging agents. We have recently been able to obtain a minimal amount of human PIASy antibody from Dr. Mary Dasso at the NIH. As a result, we used this PIASy antibody for protein profiling of various breast cancer cell lines along with other cell lines that are known to variably activate NF-κB in response to genotoxic stress agents. We utilized cellular extracts from the ER positive cell line MDA-MB-231 and the negative breast cancer cell line MCF-7 to determine if PIASy levels may dictate the ability for these cells to activate NF-κB in response to DNA damage (Figure 3). However, surprisingly we found that PIASy protein levels were much lower in the ER- line compared to the ER+ cell line. Levels were even lower than in HEK293 cells which also efficiently activate NF-κB in response to DNA damaging agents. We also observed no major correlation between cells that can efficiently activate NF-κB with DNA damaging agents and the expression of PIASy levels in the cell. This suggests that PIASy expression levels alone may not be a good predictor of whether or not a cell can activate NF-κB with DNA damaging agents. However, recent work has examined the role of a protein known as PIDD in DNA damage induced NF-κB activation. Expression of a PIDD fragment (PIDD-C) resulted in enhanced NF-κB activation with the DNA damaging agents, etoposide and doxorubicin. Interestingly, expression of the PIDD-C fragment is absent in the majority of cell lines that cannot activate NF-κB by DNA damaging agents with the exception of the HeLa cell line. Although we are still confident that PIASy is still critical for DNA damage induced NF-κB activation since we have observed that knockdown of PIASy can inhibit DNA damage induced NF-κB activation in HEK293, HeLa, and CEM cell lines.

Figure 3:

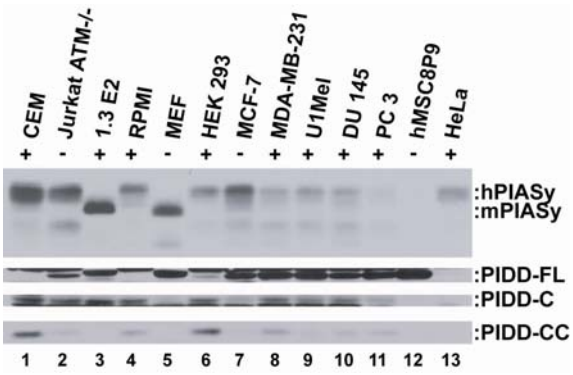

Figure 3: Cell extracts from multiple cell lines were run on a SDS-PAGE western and western blotted with anti-PIASy and anti-PIDD antibodies. Note that the Jurkat cell line is ATM deficient and this is the reason why these cell lines do not activate NF-κB with DNA damaging agents. Plus signs (+) indicate cell lines that can be activated by DNA damaging agents and minus signs (-) indicate cell lines that do not activate NF-κB by DNA damaging agents.

However, we still believe that a reduction in PIASy protein levels may sensitize ER- breast cancer cell lines to DNA damaging agents but not through mere enhanced expression as proposed previously. Interestingly, we have found that even minimal protein expression levels of PIASy protein are sufficient to activate NF-κB in response to DNA damaging agents in HEK293 cells suggesting that residual levels of PIASy in ER- breast cancer cell lines are sufficient to activate NF-κB in response to DNA damaging agents (Figure 4).

Figure 4:

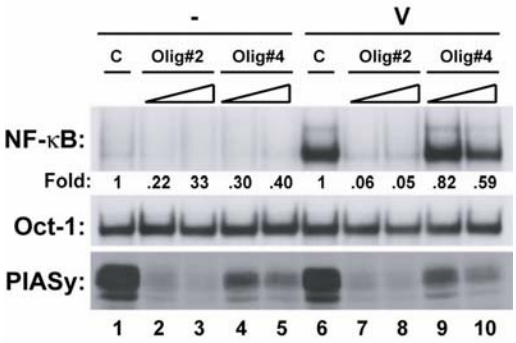

Figure 4: HEK293 cells were transfected with control (C) or two different siRNA oligos against PIASy (oligo#2 and oligo#4). Cells were treated with 10 μM VP16 for 90 minutes. Total cell extracts were made and NF-κB activity was measured by EMSA. EMSA with an Oct-1 probe was used as a control. Western blots of total protein extracts were probed with anti-PIASy antibody.

We are still working out the conditions to examine the effects of loss of PIASy protein levels on NF-κB activation in the the ER- breast cancer cell line MDA-MB-231. Since the most potent siRNA oligonucleotide (used in figure 4) gave us a modest decrease in PIASy protein levels in MDA-MB-231 cells, we are now utilizing a vector based shRNA construct that can efficiently reduce PIASy protein levels in MDA-MB-231 breast cancer cell lines that can be transfected with 80% efficiency. Studies examining its effects on basal and DNA damage induced NF-κB activation and its consequence on cell survival are currently under way. Moreover, we initially proposed that PIASy levels in MCF-7 cells exposed to antiestrogens (such as tamoxifen) will be examined to establish a link between ERα and PIASy expression. These experiments are currently being employed to see if tamoxifin may regulate PIASy expression levels.

III. Key Research Accomplishments and Conclusions:

1. Complete recombinant *in vitro* SUMOylation of NEMO

2. Mouse monoclonal antibodies against human PIASy
3. Identification of a new PIASy interacting partner, RSK1
4. Protein expression profiling of PIASy in ER+ and negative cell lines

IV. Reportable Outcomes

1. *Manuscript:* PIASy MEDIATES NEMO SUMOYLATION AND NF-κB ACTIVATION IN RESPONSE TO GENOTOXIC STRESS, Nat Cell Biol. 2006 Sep:8(9):986-93
2. *Manuscript:* SUMO AND NF-KAPPAB TIES, Cell Mol Life Sci. 2007 Aug;64(15):1979-96. Review
3. *Abstract:* A SUMO E3 PROMOTES NEMO SUMOYLATION AND NF-κB ACTIVATION IN RESPONSE TO GENOTOXIC STRESS. Keystone Symposia: NF-κB: 20 Years on the Road from Biochemistry to Pathology, 2006

V. Conclusions

Aim1: We have shown that PIASy can promote NEMO SUMOylation directly in a complete recombinant system. We have further generated our own PIASy antibody since there are no reliable commercially available antibodies. Our PIASy antibody will be used in future studies to examine novel proteins in complex with PIASy. We have further identified a new protein, RSK1 as an interacting partner for PIASy and are currently assessing its role in PIASy regulation and DNA damage induced NF-κB signaling.

Aim2: We have analyzed protein extracts from multiple cell lines including the ER+ cell line, MCF7 and the ER- cell line MDA-MB-231. We have found initially that there is no correlation between expression levels of PIASy and whether or not these cell lines can activate NF-κB in response to DNA damaging agents. We have however shown that the formation of a PIDD fragment, PIDD-CC recently implicated in DNA damage induced NF-κB activation is absent in the MCF7 cell line but not the MDA-MB-231 cell line suggesting that PIDD expression levels may play a role in whether these cell lines activate NF-κB with DNA damaging agents. We have also shown that just a residual amount of PIASy is sufficient to activate NF-κB with DNA damaging agents in HEK293 cells.

Appendices: First author published Nature Cell Biology paper and first author published CMLS review, see attached.

nature cell biology

PIASy mediates NEMO sumoylation and NF-κB activation in response to genotoxic stress

Angela M. Mabb[1], Shelly M. Wuerzberger-Davis[1] and Shigeki Miyamoto[1,2]

Protein modification by SUMO (small ubiquitin-like modifier) is an important regulatory mechanism for multiple cellular processes[1,2]. SUMO-1 modification of NEMO (NF-κB essential modulator), the IκB kinase (IKK) regulatory subunit, is critical for activation of NF-κB by genotoxic agents[3]. However, the SUMO ligase, and the mechanisms involved in NEMO sumoylation, remain unknown. Here, we demonstrate that although small interfering RNAs (siRNAs) against *PIASy* (protein inhibitor of activated STATγ) inhibit NEMO sumoylation and NF-κB activation in response to genotoxic agents, overexpression of PIASy enhances these events. PIASy preferentially stimulates site-selective modification of NEMO by SUMO-1, but not SUMO-2 and SUMO–3, *in vitro*. PIASy–NEMO interaction is increased by genotoxic stress and occurs in the nucleus in a manner mutually exclusive with IKK interaction. In addition, hydrogen peroxide (H_2O_2) also increases PIASy–NEMO interaction and NEMO sumoylation, whereas antioxidants prevent these events induced by DNA-damaging agents. Our findings demonstrate that PIASy is the first SUMO ligase for NEMO whose substrate specificity seems to be controlled by IKK interaction, subcellular targeting and oxidative stress conditions.

We previously showed that SUMO-1 modification of NEMO–IKKγ is required for the activation of NF-κB in response to etoposide (VP16) and camptothecin (CPT), but not by lipopolysaccharide or tumor necrosis factor α (TNFα)[3]. This sumoylation is associated with nuclear accumulation of IKK-free NEMO and is followed by phosphorylation on Ser 85 by ATM (ataxia telangiectasia mutated), which is required for activation of IKK and NF-κB in the cytoplasm[4]. To identify a SUMO ligase critical for NEMO, an siRNA screen was performed against known SUMO ligases. HEK293 cells transfected with siRNAs were treated with VP16 and lysates were analysed for NF-κB activation by electrophoretic mobility shift assay (EMSA). To evaluate the specificity of siRNAs, total RNA was also isolated and RT–PCR was performed for *SUMO E3* mRNAs. This analysis demonstrated minimal off-target effects for the *SUMO E3* siRNAs used (Fig. 1a).

Only *PIASy* siRNAs exhibited inhibition of NF-κB by VP16, but not TNFα (Fig. 1b, and see Supplementary Information, Fig. S1a). SiRNAs against Ran-binding protein 2 (*RanBP2*), another SUMO E3 (ref. 5), did not inhibit NF-κB activation by VP16 (data not shown). The use of two individual *PIASy* siRNA oligonucleotides showed a dose dependent decrease in PIASy protein levels that correlated with reductions in NF-κB activation (Fig. 1c). *PIASy* siRNAs also inhibited NF-κB activation by other DNA damaging agents, including CPT, ionizing radiation, doxorubicin and ultraviolet irradiation (Fig. 1d), and in HeLa human cervical cancer cells and CEM human T leukemic cells (data not shown). *PIASy* siRNAs also caused inhibition of NF-κB-dependent luciferase reporter gene activity to the same extent as those against *Ubc9*, the gene encoding the only known SUMO E2 (Fig. 1e). Combination of *PIASy* and *Ubc9* siRNAs did not further reduce reporter activity (Fig. 1e). Finally, *PIASy* siRNAs also prevented NF-κB-dependent induction of endogenous target genes, such as *IL-8* and *IκBα* in HEK293 cells (Fig. 1f and see Supplementary Information, Fig. S1b, c) and *p21* (ref. 6) in CEM cells (see Supplementary Information, Fig. S1d).

As anticipated, *PIASy* siRNAs prevented NEMO sumoylation induced by VP16 (Fig. 2a). They did not interfere with the activation of ATM, as measured by the decrease in the reactivity to phospho-Ser 1981-ATM, a specific antibody[7] (Fig. 2b). Consistent with Ser 85 phosphorylation being downstream of NEMO sumoylation[4], *PIASy* siRNAs blocked this phosphorylation event (Fig. 2c). Overexpression of PIASy resulted in both increased basal and VP16-inducible NEMO sumoylation (Fig. 2d) and NF-κB activation (Fig. 2e). In contrast, overexpression of a catalytically inactive mutant PIASy, ($PIASy^{CA}$ with the catalytic Cys 342 and Cys 347 mutated to alanine)[8] inhibited VP16-inducible sumoylation of NEMO and NF-κB activation (Fig. 2f, g).

To examine whether PIASy can function as a direct SUMO E3 for NEMO, amino-terminally HA-tagged NEMO substrate was *in vitro* translated in rabbit reticulocyte extracts in the presence of ^{35}S-methionine and then incubated with sumoylation reaction mixtures containing recombinant sumoylation components. Of the four major bands translated (see Supplementary Information, Fig. S2a), only the slowest migrating band could be immunoprecipitated with an anti-HA antibody. Thus, this band represents the full-length HA–NEMO protein,

[1]Program in Molecular and Cellular Pharmacology, Department of Pharmacology, University of Wisconsin, Madison, WI 53706, USA.
[2]Correspondence should be addressed to S.M. (e-mail: smiyamot@wisc.edu)

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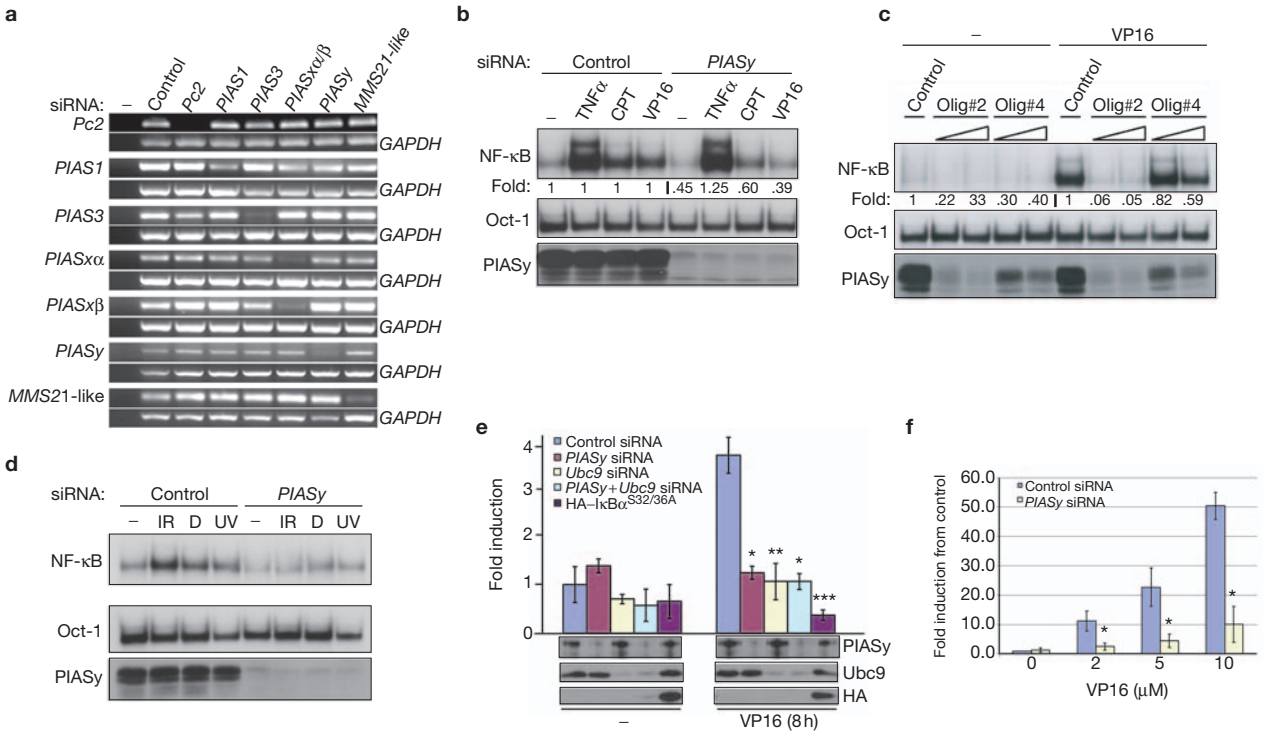

Figure 1 PIASy is necessary for NF-κB activation by genotoxic agents. (**a**) HEK293 cells were transfected with control or siRNAs against genes encoding different SUMO ligases. RNA was isolated and the expression level of mRNA for each SUMO ligase was determined using RT–PCR. GAPDH served as a loading control. (**b**) HEK293 cells were transfected as stated above with control or *PIASy* siRNAs. Cells were treated with 10 ng ml^{-1} TNFα for 15 min, 10 μM VP16 for 90 min and 10 μM CPT for 120 min. Total cell extracts were made and NF-κB activity was measured by EMSA. EMSA with an Oct-1 probe was used as a control. Western blots of total protein extracts were probed with anti-PIASy antibody. (**c**) HEK293 cells were transfected with control or two different siRNA oligonucleotides against *PIASy* (oligo #2 and oligo #4). Cells were treated with 10 μM VP16 for 90 min. Total cell extracts were analysed as in **b**. (**d**) HEK293 cells were transfected as stated in **a**. Cells were treated with 20 Gy of ionizing radiation (IR) for 90 minutes, 25 μM doxorubicin (D) for 105 minutes and 60 J m^{-2} UV (254 nm) for 135 minutes. Total cell extracts were analysed as in **b**. (**e**) HEK293 cells were transfected twice with control siRNA, *PIASy* siRNAs, *Ubc9* siRNAs, or the super-repressor IκBα$^{S32/36A}$ along with 3× *κB–Luc NF-κB* reporter gene and 24 h after the second transfection cells were treated with VP16 for 8 h. The cell extracts were used for the luciferase assay and relative luciferase activities were plotted by comparing fold induction with untreated control. Western blots of the same cell extracts with anti-PIASy, anti-Ubc9,and anti-HA antibodies are also shown to demonstrate knockdown efficiency. Means ± s.d. are shown (n = 3). Mean values were compared to the VP16-treated control siRNA using an unpaired t-test and determined to be statistically significant. *, P <0.04; **, P <0.03; ***, P <0.01. (**f**) HEK293 cells were transfected with control or *PIASy* siRNAs and treated as stated above with increasing doses of VP16. RNA was analysed for *IL-8* expression using quantitative real time RT–PCR. Means ± s. d. are shown (n = 4). Median values were compared to the VP16-treated control siRNA using the Mann-Whitney Rank Sum test and determined to be statistically significant. *, P <0.03. An uncropped scan of the top gel in **b** is shown in the Supplementary Information, Fig. S5a.

whereas the smaller bands may represent translation products derived from internal methionines or degradation products. After the sumoylation reaction, three major slower-migrating bands were observed (see Supplementary Information, Fig. S2b), of which the two highest bands could be immunoprecipitated with an anti-HA antibody. These bands also reacted with anti-NEMO and anti-SUMO-1 antibodies when analysed by western blotting (data not shown), indicating that they are sumoylated full-length NEMO species. The nature of the third band could not be determined by these analyses.

When Flag–PIASy expressed in and immunopurified from HEK293 cells was added to the *in vitro* sumoylation reaction under the conditions in which the sumoylation of NEMO was minimal with only E1 and E2 (Fig. 3a), an enhancement of NEMO sumoylation was observed that was dependent on all components of the reaction mixture. Similarly prepared PIASy^{CA} failed to enhance NEMO sumoylation. Quantification of enhancement in repeated experiments demonstrated a 2.5-fold enhancement by PIASy, but not by PIASy^{CA}.

In accordance with the immunopurified PIASy described above, recombinant human His–PIASy purified from *Escherichia coli* showed enhancement of NEMO sumoylation *in vitro* (not shown). However, we were unable to purify it to near homogeneity. Thus, a recombinant *Xenopus* PIASy protein that shares 77% sequence identity and 84% homology with the human protein (His–xPIASy, Fig. 3d) and that was previously described for *in vitro* sumoylation of topoisomerase II, was used[9]. His–xPIASy, purified to near homogeneity, enhanced sumoylation of *in vitro* translated NEMO, which showed major bands similar to those observed with FLAG–PIASy (Fig. 3c), as well as high molecular weight smears or ladders. All modified NEMO species could be cleaved by the catalytic domain of recombinant SENP1, a SUMO protease (Fig. 3c). Importantly, His–xPIASy promoted SUMO-1 isoform-specific modification of NEMO, similar to genotoxic stress conditions *in vivo*[3], as it failed to efficiently sumoylate NEMO in the presence of SUMO-2 or SUMO-3 (see Supplementary Information, Fig. s2e). A similar result was obtained using His–NEMO protein expressed in *E. coli* and purified to near homogeneity (Fig. 3d, e). Thus, these results demonstrated that PIASy enhanced SUMO-1 modification of NEMO in a highly purified *in vitro* system. In addition, overexpression of *Xenopus* PIASy could enhance NF-κB activation in HEK293 cells and could also

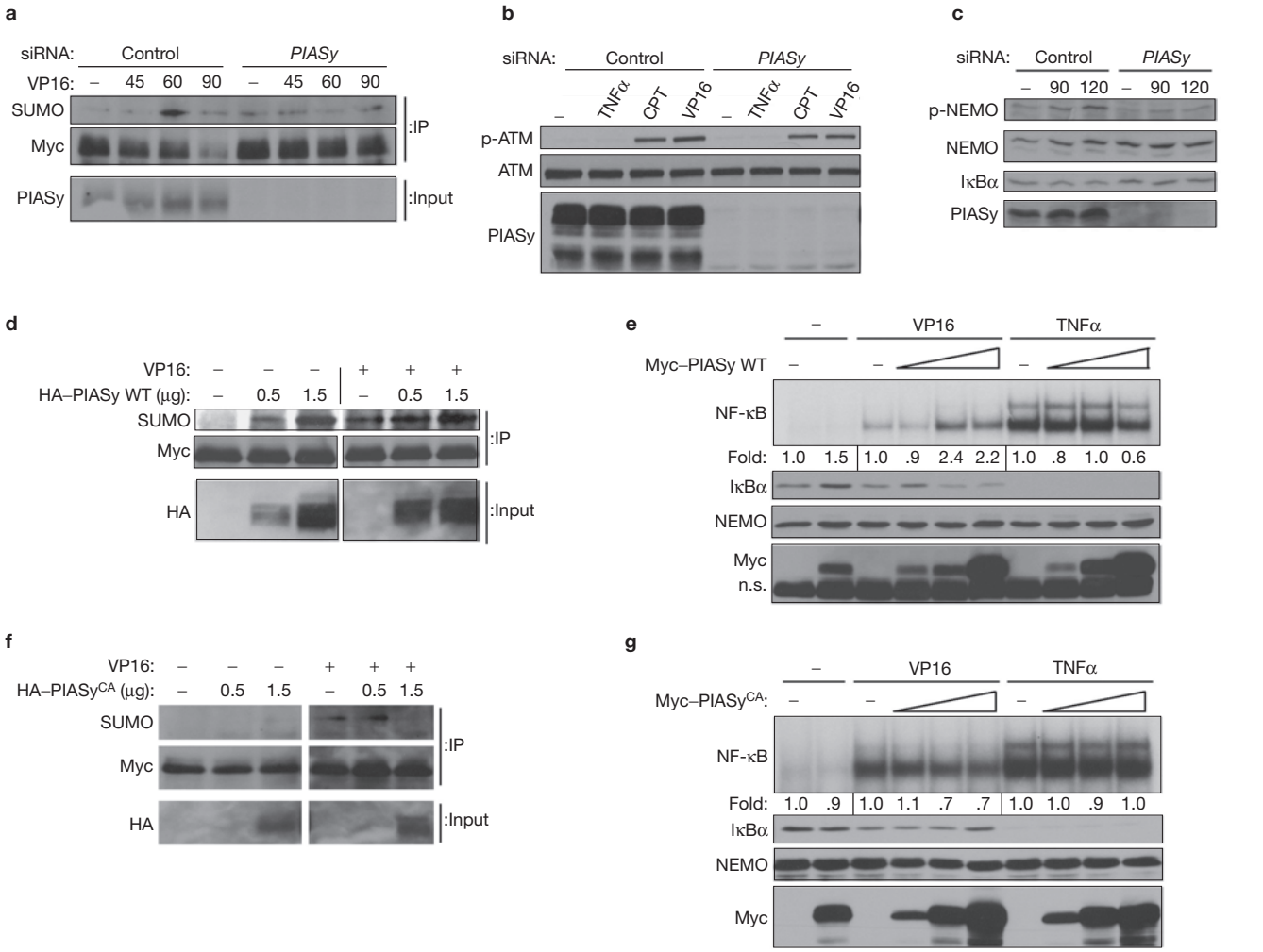

Figure 2 PIASy modulates NEMO sumoylation and NF-κB activation in response to genotoxic stress. (**a**) HEK293 Myc–NEMO stable cells were transfected with control or *PIASy* siRNAs. Cells were left untreated or treated with 10 μM VP16 for indicated times in minutes. Cell lysates were boiled in 1% SDS and 10% input samples were taken. The remaining samples were then diluted to 0.1% SDS and immunoprecipitated with anti-Myc antibody. The precipitates were blotted with anti-SUMO-1 and anti-Myc antibodies. Inputs were blotted with anti-PIASy antibody. (**b**) HEK293 cells were transfected with control or *PIASy* siRNAs and treated with 10 ng ml^{-1} TNFα for 15 min, 10 μM CPT for 75 min or 10 μM VP16 for 60 min. Total cell extracts were examined by immunoblotting with an anti-ATM and anti-phospho-Ser 1981-ATM antibodies. PIASy protein was examined by immunoblotting with an anti-PIASy antibody. (**c**) HEK293 cells were transfected as stated in **b** and treated with 10 μM VP16 for the indicated times. Total cell extracts were examined by immunoblotting with an anti-phospho-Ser 85-NEMO, anti-NEMO, anti-IκBα and anti-PIASy antibodies. (**d**) HEK293 Myc–NEMO stable cells were transfected with 0.5 and 1.5 μg of a PIASy expression vector. Cells were left untreated or treated with 10 μM VP16 for 60 min. Cell lysates were prepared and immunoprecipitated as in **a**. The precipitates were blotted with anti-SUMO-1 and anti-Myc antibodies. 10% inputs were blotted with anti-HA antibody. (**e**) HEK293 cells were transfected with 0.5, 1.5 and 4.0 μg of a PIASy expression vector. Cells were treated with 10 μM VP16 for 60 min and 10 ng ml^{-1} TNFα for 15 min. Total cell extracts were measured for NF-κB activity by EMSA. Cell lysates were probed with anti-IκBα, anti-NEMO and anti-Myc antibodies. n.s., nonspecific band. (**f**) HEK293 Myc–NEMO stable cells were transfected with a $PIASy^{CA}$ expression vector. Cells were processed as in **a**. Precipitates were blotted as in **d**. (**g**) HEK293 cells were transfected with 0.5, 1.5 and 4.0 μg of a $PIASy^{CA}$ expression vector. Cells were analysed as in **e**. An uncropped scan of the top gel in **a** is shown in the Supplementary Information, Fig. S5b.

partially rescue the *PIASy* siRNA-induced inhibition of NF-κB activation in response to VP16 (see Supplementary Information, Fig. 2c, d), indicating that *Xenopus* PIASy can functionally compensate for endogenous human PIASy in a mammalian cellular environment.

NEMO sumoylation in response to genotoxic agents is blocked by mutations of Lys 277 and Lys 309 (ref. 3). To determine whether PIASy could promote sumoylation on these residues, sumoylation assays were performed using $NEMO^{K277A}$ and $NEMO^{K309A}$ mutants in the presence of His–xPIASy (Fig. 3f, g). Whereas K277A mutagenesis prevented the formation of one of the sumoylated bands (open circle) and caused an apparent increase in sumoylation of another band (closed circle), $NEMO^{K309A}$ lost the slower migrating of the two sumoylated bands (closed circle). These results suggested that the upper of the two bands represented sumoylated NEMO on Lys 309 and the lower band on Lys 277. When both lysines were mutated ($NEMO^{DK}$), the two bands migrated differently from those seen in the wild-type NEMO protein (single and double stars). Thus, it is likely that mutation of Lys 277 and Lys 309 results in sumoylation of NEMO on alternative sites. Although these lysine residues conform to the optimal sumoylation site, ψKxE (where ψ represents a hydrophobic amino acid and x represents any amino acid)[10,11], both of these sites partially deviate from this sequence due to the presence of alanine at position 276 and aspartate at

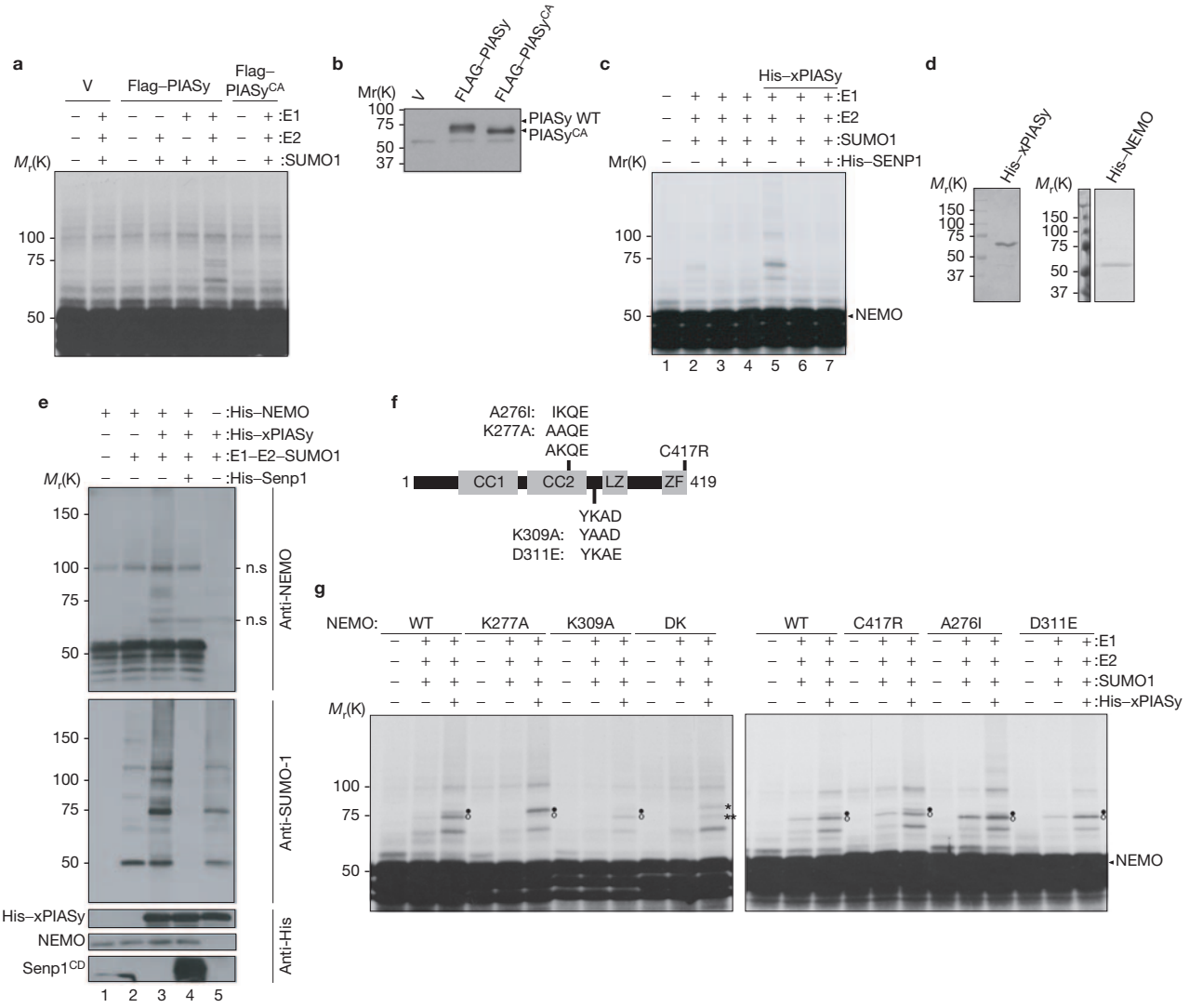

Figure 3 PIASy promotes sumoylation of NEMO *in vitro*. (**a**) HEK293 cells were transfected with Flag–PIASy WT and CA constructs or vector control (V). Cell lysates were immunoprecipitated with an anti-Flag antibody and washed extensively using high salt buffer. Immunopurified PIASy WT and CA beads were then added to the *in vitro* sumoylation assay containing *in vitro* translated ^{35}S HA–NEMO, and the reaction mixtures were incubated at 30 °C for 90 min. Reaction products were separated by SDS–PAGE and the dried gel was exposed to an X-ray film. (**b**) Anti-Flag western blot showing immunopurified Flag–PIASy WT and CA mutant, and vector control used for the *in vitro* assay in **a**. (**c**) *In vitro* sumoylation assay was performed with *in vitro* translated ^{35}S HA–NEMO and 0.5 μg of recombinant His–xPIASy for 75 min at 30 °C. Recombinant His–SENP1 (0.75 and 1.5 μg in lanes 6 and 7, respectively) was added after *in vitro* sumoylation to cleave the SUMO-1 moiety from modified NEMO. Reactions were terminated in 2× SDS sample buffer, run on an SDS–PAGE gel, fixed and exposed to film. (**d**) Commassie stained gels of recombinant *Xenopus* His–xPIASy and recombinant human His–NEMO. (**e**) *In vitro* sumoylation assay was performed using 0.75 μg of recombinant His–NEMO and 1 μg of recombinant His–xPIASy at 30 °C for 75 min. His–SENP1 was added after sumoylation in lane 4. Samples were terminated in 2× SDS sample buffer, run on an SDS–PAGE gel and blotted with anti-NEMO, anti-SUMO-1 and anti-His antibodies. (**f**) Schematic representation of the NEMO mutants used in **g**. CC1, coiled-coil 1; CC2, coiled-coil 2; LZ, leucine zipper; ZF, zinc finger. The positions of mutated amino-acid residues are indicated. (**g**) *In vitro* sumoylation assay was performed as in **a** with the addition of *in vitro* translated ^{35}S-methionine labelled HA–NEMO mutants K277A, K309A, K277A/K309A (DK), C417R, A276I and D311E and using recombinant His–xPIASy as in **c**. Closed circle indicates SUMO-1 modification of NEMO putatively at Lys 309, open circle indicates SUMO-1 modification of NEMO putatively at Lys 277 and * and ** represent SUMO-1 modification of NEMO at unidentified sites.

position 311 (Fig. 3f). To further examine site-selective sumoylation *in vitro*, Ala 276 was mutated to isoleucine to create a more hydrophobic residue at the –1 position. This resulted in increased sumoylation of the putative Lys 277 band compared with wild-type NEMO (Fig. 3g). In contrast, when Asp 311 was mutated to glutamate, sumoylation of the Lys 309 band was not greatly affected, suggesting that PIASy promotes sumoylation of NEMO at Lys 309 equivalently with either aspartate or glutamate at the +2 position. Mutations within the carboxy-terminal zinc finger domain of NEMO severely hinder NEMO sumoylation and NF-κB activation by genotoxic stress inducers[3]; however, a NEMO zinc-finger mutant (NEMO^{C417R}) was efficiently sumoylated by PIASy (Fig. 3g). Hence, the intact zinc finger is not necessary for direct NEMO sumoylation by PIASy. Similar results were obtained for all of the NEMO mutants with immunopurified human Flag–PIASy (see Supplementary Information, Fig. 3a).

To test whether PIASy interacts with NEMO *in vivo*, HA–PIASy was transfected into HEK293 cells and endogenous NEMO was immunoprecipitated following exposure to VP16. A modest amount of HA–PIASy

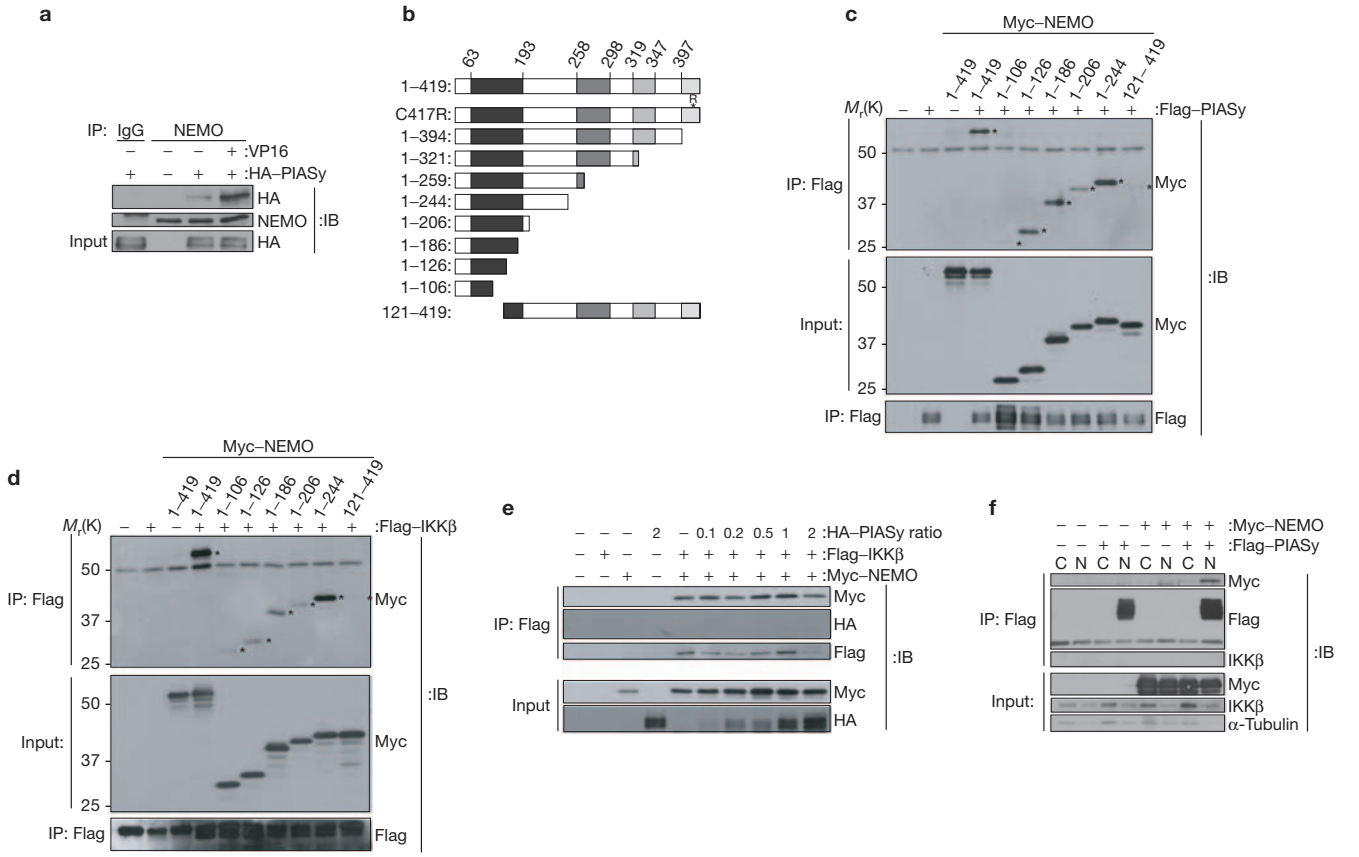

Figure 4 NEMO interacts with PIASy in a manner that is mutally exclusive with IKKβ binding. **(a)** HEK293 cells were transfected with HA–PIASy or empty vector. Lysates were immunoprecipitated with anti-NEMO antibody or a mouse IgG control. Samples were immunoblotted with anti-HA and anti-NEMO antibodies. (**b**) Schematic representation of NEMO mutants used for coimmunoprecipitation analysis with human Flag–PIASy and human Flag–IKKβ. Amino acids 63–193 represent coiled-coil 1 (CC1), 258–298 represent coiled-coil 2 (CC2), 319–347 represent a leucine zipper and 397–418 represent a zinc finger. (**c**) HEK293 cells were transfected with Flag–PIASy and Myc–NEMO mutants. Cells extracts were immunoprecipitated with an anti-Flag antibody and probed with an HRP-conjugated anti-Myc antibody. 2% input was also probed with anti-Myc antibody. (**d**) HEK293 cells were transfected with Flag-IKKβ and Myc–NEMO mutants. Extracts were immunoprecipitated as in **c**. (**e**) HEK293 cells were transfected with Flag-IKKβ, Myc–NEMO and increasing amounts of HA–PIASy construct. Cell extracts were immunoprecipitated with an anti-Flag antibody and probed with anti-Flag and HRP-conjugated anti-HA and anti-Myc antibodies. Immunoblots with 2% inputs are also shown. (**f**) HEK293 cells were transfected with Flag–PIASy, Myc–NEMO or empty vector. Cytoplasmic and nuclear extracts were isolated and PIASy was immunoprecipitated with anti-Flag antibody. Samples were immunoblotted with anti-Flag, anti-Myc, anti-IKKβ and anti-tubulin antibodies.

was present in NEMO precipitates without any stimulation (Fig. 4a and see Supplementary Informtaion, Fig. S3b) and this interaction was further augmented on treatment with VP16. Myc–NEMO mutants (Fig. 4b) and Flag–PIASy were co-expressed and their interactions were examined to determine the region(s) of NEMO that are critical for PIASy interaction. Successive C-terminal deletions demonstrated that NEMO$^{1-126}$ was sufficient to interact with PIASy (Fig. 4c and see Supplementary Information, Fig. S3c). A deletion of the first 120 amino-acid residues of NEMO abrogated its interaction with PIASy. These results showed that the amino terminus of NEMO is both necessary and sufficient for PIASy association.

The N-terminal region of NEMO is also essential for IKKβ interaction[12]. Coimmunoprecipitation experiments with NEMO mutants and Flag–IKKβ demonstrated that association of the NEMO mutants with IKKβ was nearly identical to interaction with PIASy (Fig. 4d). Cotransfection and coimmunoprecipitation experiments further demonstrated that NEMO–PIASy and NEMO–IKK interactions are mutually exclusive (Fig. 4e and see Supplementary Information, Fig. S3d). Moreover, subcellular fractionation studies revealed a NEMO–PIASy complex in the nuclear fraction, but the majority of IKKβ was located in the cytoplasm (Fig. 4f). Even though a small amount of IKKβ was observed in the nuclear fraction under these conditions, an IKKβ–PIASy interaction was not detected. These results suggested that PIASy–NEMO interaction occurs predominantly in the nucleus in a manner mutually exclusive with NEMO–IKKβ interaction.

As many double-strand break (DSB)-inducing agents also induce oxidative stress[13–16], we examined whether such a stress could be critical for NEMO sumoylation and NF-κB activation. The antioxidants N-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) prevented sumoylation of endogenous NEMO and NF-κB activation without interfering with ATM activation in response to VP16 (Fig 5a, b) and CPT (data not shown) in CEM cells. Moreover, the pro-oxidant H_20_2 was capable of promoting NEMO sumoylation, which was sensitive to inhibition by antioxidants (Fig. 5c). H_20_2 also promoted PIASy–NEMO interaction (Fig. 5d). Taken together, these findings suggested the possibility that oxidative stress may be critical for induction of a PIASy–NEMO interaction and NEMO sumoylation after exposure to certain DNA-damaging agents.

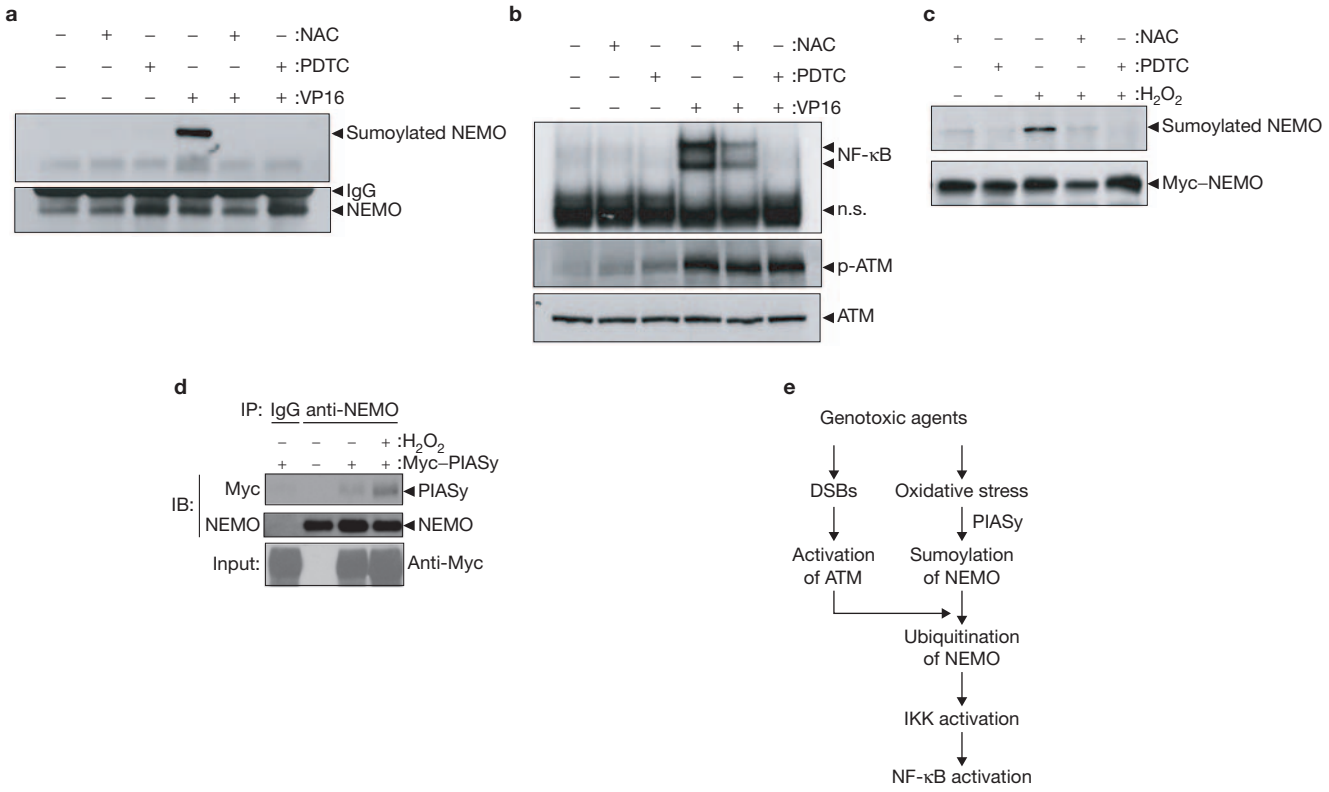

Figure 5 Oxidative stress seems to be required for NEMO sumoylation in response to VP16. **(a)** CEM cells were pretreated for 3 h with 50 mM NAC and for 30 min with 100 μM PDTC. Cells were then treated for 1 h with 10 μM VP16. Cell lysates were boiled in 1% SDS and then diluted to 0.1% SDS and immunoprecipitated with anti-NEMO antibody. Precipitates were immunoblotted with anti-SUMO-1 and anti-NEMO antibodies. (**b**) CEM cells were treated as above and cell lysates were analysed by EMSA and western blotted with an anti-ATM and anti-phospho-Ser 1981-ATM antibodies. (**c**) 1.3E2 NEMO-deficient murine pre-B cells stably expressing Myc–NEMO were pretreated with NAC and PDTC as above. Cells were treated with 2mM H_2O_2 for 45 min. Cell lysates were immunoprecipitated with anti-Myc antibody and precipitates were immunoblotted with anti-SUMO-1 and anti-Myc antibodies. (**d**) HEK293 cells were transfected with Myc–PIASy and 24 h later they were treated with 2 mM H_2O_2 for 45 min. Cell lysates were immunoprecipitated with an anti-NEMO antibody and the precipitates were immunoblotted with HRP-conjugated anti-Myc and anti-NEMO antibodies. (**e**) Schematic representation of a model for genotoxic stress-induced NF-κB activation. Genotoxic agents cause both DSBs and oxidative stress. DSBs lead to ATM activation, whereas oxidative stress may promote NEMO–PIASy interaction and NEMO sumoylation. These parallel events together promote NEMO ubiquitination and activation of IKK and NF-κB. An uncropped scan of the top gel in **a** is shown in the Supplementary Information, Fig. S5c.

In summary, we have shown that PIASy meets the minimal requirements of a SUMO ligase for NEMO in the genotoxic stress-induced NF-κB signalling pathway. First, a reduction of the expression of endogenous PIASy by siRNAs reduces the cellular capacity to cause SUMO-1 modification of NEMO and activate NF-κB in response to several DNA-damaging agents. Reduction of PIASy expression also attenuates NF-κB-dependent induction of both reporter and endogenous target genes without interfering with ATM activation. Second, overexpression of wild-type PIASy augments both basal and genotoxic stress-inducible sumoylation of NEMO and NF-κB activation. In contrast, a catalytically inactive PIASy mutant blocks NEMO sumoylation and NF-κB activation in response to genotoxic stimuli. Third, purified recombinant PIASy promotes modification of NEMO by SUMO-1, but not SUMO-2 or SUMO-3. *In vitro* sumoylation of NEMO by PIASy is also sensitive to mutations of Lys 277 and Lys 309, residues that are also necessary for *in vivo* sumoylation in response to genotoxic stress. Fourth, PIASy inducibly interacts with NEMO in response to DNA-damaging agents *in vivo*. Thus, these studies identify PIASy as the first SUMO E3 for NEMO relevant to the genotoxic stress NF-κB signalling pathway (Fig. 5e).

Our study also provides additional unexpected observations. A previous study using an RNAi screen against all putative ubiquitin proteases present in the human genome identified only CYLD (cylindromatosis tumor suppressor protein) as functioning in cytokine signalling[17]. Similarly, our RNAi screen against *SUMO E3s* found that only siRNAs against *PIASy* had inhibitory effects on NF-κB activation by genotoxic stress inducers. In contrast, some of the other E3s tested showed reproducible increases in NF-κB activation (for example, PIAS1 and PIAS3). It is unclear how these siRNAs cause this increase, but PIAS1 and PIAS3 have been shown to cause inhibition of p65 DNA binding activity[18,19]. Although we cannot exclude the possibility that other SUMO E3s also contribute to NEMO sumoylation in specific cellular contexts, our study demonstrates that PIASy is critical for NF-κB activation by genotoxic agents in multiple cell types.

In addition, we found that the N-terminal domain of NEMO is both necessary and sufficient for PIASy interaction. Interestingly, this overlaps with the region of NEMO where IKKβ associates[12]. IKKβ and PIASy fail to coimmunoprecipitate in the presence of NEMO, suggesting that NEMO–PIASy and NEMO–IKKβ complexes are mutually exclusive. The interaction between NEMO and PIASy is largely nuclear, as opposed to the IKKβ–NEMO interaction that is mostly cytoplasmic[12]. Thus, cell compartmentalization is an additional means to segregate these complexes. Our previous study indicated that IKK-free NEMO

becomes sumoylated and SUMO-modified NEMO accumulates in the nucleus[3]. The mutually exclusive interactions between NEMO–IKKβ and NEMO–PIASy partly explain why only IKK-free NEMO is targeted for sumoylation *in vivo*.

Nuclear interaction of PIASy–NEMO and increased basal sumoylation and NEMO nuclear accumulation (data not shown) on PIASy overexpression also suggest that NEMO sumoylation may occur in the nuclear compartment. As SUMO–NEMO fusion proteins displayed tendencies to accumulate in the nucleus, SUMO modification was suggested to promote NEMO nuclear targeting[3]. The zinc finger domain of NEMO was required for both sumoylation and nuclear targeting, further suggesting that this domain is required for sumoylation to take place. However, our current data demonstrate that the zinc finger is not required for NEMO interaction with PIASy *in vivo* or sumoylation *in vitro*. Instead, it may be critical for events involved in oxidative-stress signalling to promote NEMO nuclear localization[4] (Fig. 5e) or PIASy interaction of NEMO that is already located in the nucleus[20]. Alternatively, the zinc-finger domain may be necessary for stabilization of sumoylated NEMO, possibly by interfering with its interaction with a SUMO protease. PIDD (p53-inducible death domain) and RIP1 (receptor interacting protein 1) may be involved in the regulation of nuclear accumulation and sumoylation of NEMO in a manner dependent on the zinc-finger domain[20]. As activation of NF-κB by a variety of anti-cancer DNA-damaging agents is linked to modulation of malignant behaviours[21,22] (including resistance to chemo- and radiation therapy), further understanding of the enzymatic components and mechanisms involved may facilitate the development of novel anti-cancer agents targeting this signalling pathway. □

METHODS

Reagents. Etoposide (VP16), camptothecin (CPT), doxorubicin, 2, 5-diphenyloxazole (PPO), phenylmethylsulphonylfluoride (PMSF), aprotinin, N-acetylcysteine, N-ethylmaleimide and iodoacetamide were obtained from Sigma (St Louis, MO). Pyrrolidinedithiocarbamate was obtained from Alexis Corporation (Lausanne, Switzerland). Human recombinant TNFα and anti-tubulin antibody were obtained from Calbiochem (La Jolla, CA). Antibodies for horseradish peroxidase (HRP)-conjugated anti-goat, IκBα, c-Myc and NEMO were obtained from Santa Cruz (Santa Cruz, CA). Anti-c-Myc and anti-HA peroxidase antibodies were obtained from Roche (Indianapolis, IN). Anti-NEMO antibody was obtained from BD Pharminogen (San Jose, CA). Anti-Flag antibody was obtained from Sigma. Anti-GMP-1 (SUMO-1) and SUMO-2 antibodies was obtained from Zymed Laboratories (San Francisco, CA). ATM antibody was purchased from Genetex (San Antonio, TX) and anti-phospho-ATM (Ser 1981) was obtained from R & D Systems (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse antibodies, Gamma Bind G-Sepharose beads and protein G–Sepharose beads were obtained from Amersham (Piscataway, NJ). SUMO-1 antibody, human recombinant SUMO-1, SUMO-2, SUMO-3, *Saccharomyces cerevisiae* SUMO E1 (Aos1p/Uba2p), human recombinant SUMO E2 (Ubc9) and human recombinant His–SENP1 catalytic domain (CD) proteins were purchased from Boston Biochem (Cambridge, MA).

Cell culture. HEK293 and CEM cells were maintained as previously described[23]. HEK293 6× Myc–NEMO and 1.3E2 6× Myc–NEMO stable cell lines were generated as previously described[24].

siRNAs, RNA isolation and RT–PCR. siRNA-mediated knockdown of polycomb 2 (*Pc2*), *PIAS1*, *PIAS3*, *PIASxα/β*, *PIASy* and *MMS21*-like was performed by calcium phosphate transfection of 200 pmol of siGENOME SMARTpool double-stranded RNA oligonucleotides (Dharmacon, Lafayette, CO) or control siRNA (Dharmacon). A second transfection was also performed after 24 h to increase RNA interference efficiency. RNA from these transfected cells was isolated using the QIAshredder and RNeasy mini kits (Qiagen, Valencia, CA) 24 h after the second transfection. Multiplex RT–PCR was performed using Qiagen's OneStep RT-PCR kit according to the manufacturers' protocol. RT–PCR primers and siRNA oligonucleotide sequences are provided in the Supplementary Information, Fig. S4.

Cloning of PIASy and NEMO wild type and mutants. Generation of pcDNA3.1(+) 6×Myc–NEMO and pcDNA3.1(+) 2×HA–NEMO was described previously[24]. His–NEMO was cloned into pET-28a(+) from Novagen (Madison, WI) using restriction sites *Hind*III and *Xho*I. PcDNA3.1(+) 2×HA–NEMO A276I and D311E mutants were generated using site directed mutagenesis from pcDNA3.1(+) 2×HA–NEMO template. pcDNA3 2×HA–NEMO K277A and K309A mutants were generated through subcloning of pcDNA3 6×Myc–NEMO K277A and K309A (ref. 3) into pcDNA3 2×HA (Invitrogen) vector using restriction sites *BamH*I and *Xba*I. Flag-, Myc- and HA-tagged full-length PIASy was cloned through PCR amplification of PIASy from ATCC (Mammalian Genome Collection, Manassa, VA; IMAGE ID: 5176540) into pFlag–CMV-2 (Sigma) using *Not*I–*Xba*I restriction sites. PIASy was cloned into pcDNA3.1(+) 6×Myc (a gift from Z. Chen, UT-Southwestern, Dallas, TX) and pcDNA3.1(+) 2×HA constructs using *BamH*I–*Eco*RI restriction sites. PIASy C342/347A (CA) mutant was generated using two-step PCR mutagenesis from the original PIASy template. Primers used for cloning are available on request. All constructs were verified by DNA sequencing. *Xenopus* pET28a–PIASy was subcloned into the pcDNA3–Myc3 vector using *Eco*RI and *Xho*I restriction sites. Generation of a Flag–IKKβ construct was previously described[24].

Western blotting, immunoprecipitation and EMSA. Cell lysates production and western blotting was performed as previously described[25]. EMSA analysis and image quantification was also performed as previously described[26]. For NEMO–PIASy immunoprecipitations, cell lysates were precleared overnight with Gamma Bind G Sepharose. Precleared lysates were then immunoprecipitated as previously described[26].

Luciferase reporter assay. HEK293 cells were transfected with 25 ng of 3×κB–Luc, 25 ng of CMV–β-Gal, and 200 pmol of *PIASy* siRNA or control siRNA as stated above. Cells were treated with 10 μM VP16 for 8 h and harvested according to Promega's luciferase assay system kit. β-galactosidase activity was measured using the Galacton-Plus kit purchased from Tropix (Bedford, MA). The transfection efficiency was normalized within each cell line with β-galactosidase activity. Results were displayed as fold induction from untreated control.

Quantitative RT–PCR analysis. HEK293 cells were transiently transfected with control and *PIASy* siRNA as stated above and treated with increasing doses of VP16 for 3 h. Generation of total RNA from treated cells, cDNA synthesis from total RNA and quantitative real-time RT–PCR was previously described[6]. PCR primers used in this study are provided in the Supplementary Information, Fig. S4. Product accumulation was monitored by SYBR green fluorescence, with the relative expression levels determined from a standard curve of serial dilutions of cDNA. All samples were normalized to GAPDH levels and results were displayed as fold induction from untreated control.

Purification of recombinant His–NEMO. His–NEMO protein was generated in BL21 Rosetta 2 strain cultured in LB broth containing 25 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol. Cultures were induced at A_{600} = 0.5 with 1 mM IPTG for 3 h at 37 °C. Cell pellets were resuspended in equilibration/wash buffer (50 mM sodium phosphate, 300 mM NaCl at pH 7.0) and were lysed by sonication (4 rounds of 10 s bursts on ice). Cell debris and inclusion bodies were removed by centrifugation. The resulting supernatant was added to BD Talon Co^{2+} metal affinity resin (BD Biosciences). Bound protein was washed three times in equilibration/wash buffer and eluted in equilibration/wash buffer containing 150 mM imidazole. His–NEMO protein was then dialysed against 50 mM Tris at pH 7.6 and 5 mM $MgCl_2$.

***In vitro* and *in vivo* sumoylation assays.** *In vitro* sumoylation of NEMO was performed by *in vitro* translation of HA–NEMO protein using the TNT T7 Coupled Reticulocyte Extract System (Promega) in the presence of Redivue ^{35}S-methionine (Amersham). *In vitro* sumoylation assays were performed using 7.5 μg ml^{-1} SUMO E1 (Aos/Uba1), 50 μg ml^{-1} SUMO E2 (Ubc9) and 50 μg ml^{-1} of SUMO-1 added to 4 μl of *in vitro* translated NEMO in the presence of an ATP regenerating system (10 mM creatine phosphate, 10 units creatine kinase, 1 unit inorganic pyrophosphatase and 2 mM ATP) in 50 mM Tris at pH 7.6 and 5 mM $MgCl_2$

in a 40 μl reaction with 1 μg ml^{-1} aprotinin and 1 mM PMSF. The reaction was incubated at 30 °C for 180 min. End products were terminated with SDS sample buffer and separated by SDS–PAGE gels. Sumoylated products were detected by fixing SDS–PAGE gels in acetic acid followed by addition of the scintillant PPO. Gels were dried and exposed to autoradiograph film (Kodak, Rochester, NY). For *in vitro* sumoylation with recombinant His–NEMO and His–xPIASy, reaction conditions were performed as stated above with the exception of using 0.75 μg His–NEMO substrate and 1 μg His–xPIASy in a 40 μl reaction. End products were treated as above except SDS–PAGE gels were transferred and immunoblotted with goat anti-NEMO, rabbit anti-SUMO and His anti-mouse antibodies. For proteolysis of SUMO modified NEMO, SUMO reaction end products (40 μl reaction total) were incubated with 10 mM DTT at 30 °C for 15 minutes. 0.75 or 1.5 μg His-$SENP1^{CD}$ was then added to samples and incubated at 30 °C for 1 h. Reactions were terminated in SDS sample buffer and reaction products separated by SDS–PAGE gels.

To determine the effects of PIASy on NEMO sumoylation *in vitro*, pFlag–CMV-2 PIASy wild-type and pFlag–CMV-2 $PIASy^{C342/347A}$ expression constructs were transiently transfected into HEK293 cells. Cell extracts were prepared from these cells 24 h following transfection in immunoprecipitation buffer (20 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA, 250 mM NaCl at pH 7.4). PIASy protein was immunoprecipitated by addition of anti-Flag antibody and Gamma Bind G-Sepharose. The immunoprecipitates were washed in high salt immunoprecipitation buffer (20 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA and 500 mM NaCl at pH 7.4) with 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM DTT, 1 μg aprotinin per ml and 0.5 mM PMSF four times, followed by washing two times with *in vitro* sumoylation buffer (50 mM Tris and 5 mM $MgCl_2$ at pH 7.6) with 1 μg aprotinin per ml and 0.5 mM PMSF. Immune purified PIASy beads (20 μl) were added to the sumoylation reaction as stated above but incubated at 30 °C for 110 min.

To detect *in vivo* sumoylation of NEMO, cells were rinsed twice with PBS, harvested and pelleted by centrifugation. The cell pellets were then lysed in an immunoprecipiation buffer (20 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA and 250 mM NaCl at pH 7.4) in the presence of 1% SDS, protease and phosphatase inhibitors[24], as well as 10 mM N-ethylmaleimide and 3 mM iodoacetamide. Samples were then boiled for 20 min to denature proteins and spun down to remove cellular debris and inputs from this lysate were taken. Samples were then diluted to 0.1% SDS with immunoprecipitation buffer. Myc–NEMO or endogenous NEMO was immunoprecipitated with anti-Myc or anti-NEMO antibody, respectively, on Protein G–Sepharose beads overnight and washed with immunoprecipitation buffer four times. Final immunoprecipitates were boiled in SDS sample buffer, separated by SDS–PAGE gels, transferred and immunoblotted as previously described[25].

Cell fractionation. Briefly, nuclear and cytoplasmic extracts were generated through hypotonic shock followed by nuclei extraction with salt as previously described[25].

Statistical analysis. Statistical analysis was performed with SigmaStat 3.0 software (SYSTAT Software, Inc., Richmond, CA). Mean values with equal variance were compared using and unpaired *t*-test. Mean values with unequal variance were compared using the Many-Whitney Rank Sum test.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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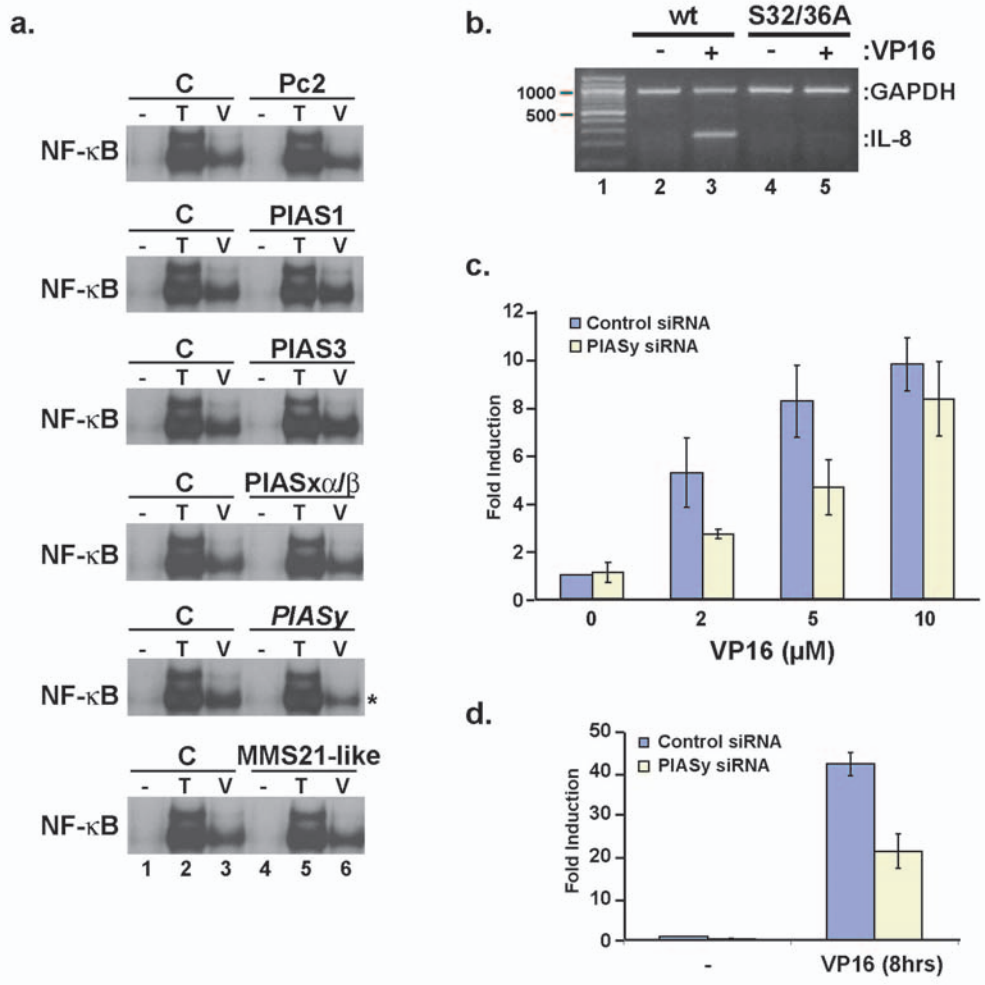

Figure S1: **a.** HEK293 cells were transfected with control or siRNAs against different SUMO ligases. Cells were treated with 10 μM VP16 for 60 minutes or 10ng/mL TNFα for 15 minutes. Total cell extracts were made and NF-κB activity was measured using EMSA. **b.** HEK293 and HEK293 IκBα-S32/36A stable cells were treated with 10 μM VP16 for 3 hours. RNA from cells was isolated and RT-PCR was performed using IL-8 and GAPDH primers. **c.** HEK293 cells were transfected with control or PIASy siRNAs and treated with increasing doses of VP16 for 3 hours. RNA was isolated and analyzed for IκBα using quantitative real time RT-PCR. **d.** CEM cells were transfected with control or PIASy siRNAs and treated with 10 μM VP16 for 6 hours. Samples were analyzed for p21 using quantitative real time RT-PCR.

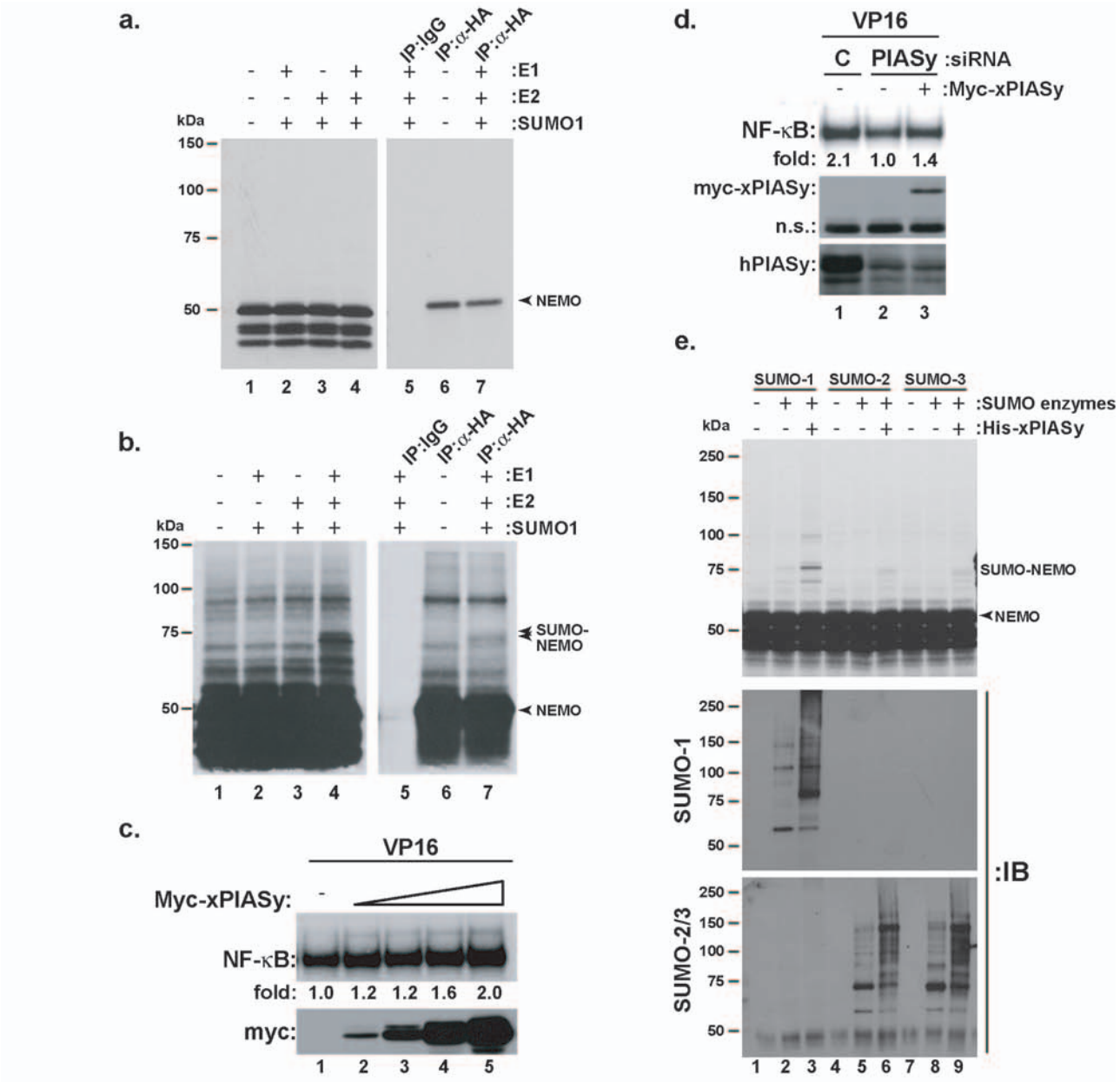

Figure S2: **a.** Light exposure of *in vitro* translated NEMO assay peformed in b. **b.** *In vitro* translated ^{35}S-methionine-labeled HA-NEMO was added to *in vitro* SUMOylation reaction. SUMO E1 and E2 were added as stated in materials and methods. Samples were run on an SDS-PAGE gel, fixed, and exposed to film. Alternatively, reaction products were boiled in 1%SDS and immunoprecipitated with an anti-HA antibody and analyzed as stated above. **c.** HEK293 cells were transfected with 0.15, 0.5, 1.5, and 4.0 µg of *Xenopus* PIASy (Myc-xPIASy) DNA. Cells were treated with 10 µM VP16 for 75 minutes. Total cells extracts were measured for NF-κB activity by EMSA. **d.** HEK293 cells were transfected with PIASy siRNA (oligo#2) and 0.2 µg Myc-xPIASy DNA. Cells were treated with 10 µM VP16 for 120 minutes. NF-κB activity was measured as in c. **e.** *In vitro* translated ^{35}S-methionine-labeled HA-NEMO was added to SUMO reaction as in a. Reactions were performed in the presence of different SUMO protein isoforms: SUMO-1 (lanes 1-3), SUMO-2 (lanes 4-6), or SUMO-3 (lanes 7-9). 0.5 µg of His-xPIASy was added to the SUMO reaction. The mixture was incubated at 30 °C for 75 minutes and terminated in 2xSDS sample buffer. Samples were run on an SDS-PAGE gel, fixed, and exposed to film. Alternatively, terminated reaction end products were western blotted and probed with mouse anti-SUMO-1 and rabbit anti-SUMO-2 (which also recognizes SUMO-3), showing that His-xPIASy can promote modification of reticulocyte proteins by SUMO-1, -2 and –3 even though it preferentially promotes SUMO-1 modification of NEMO.

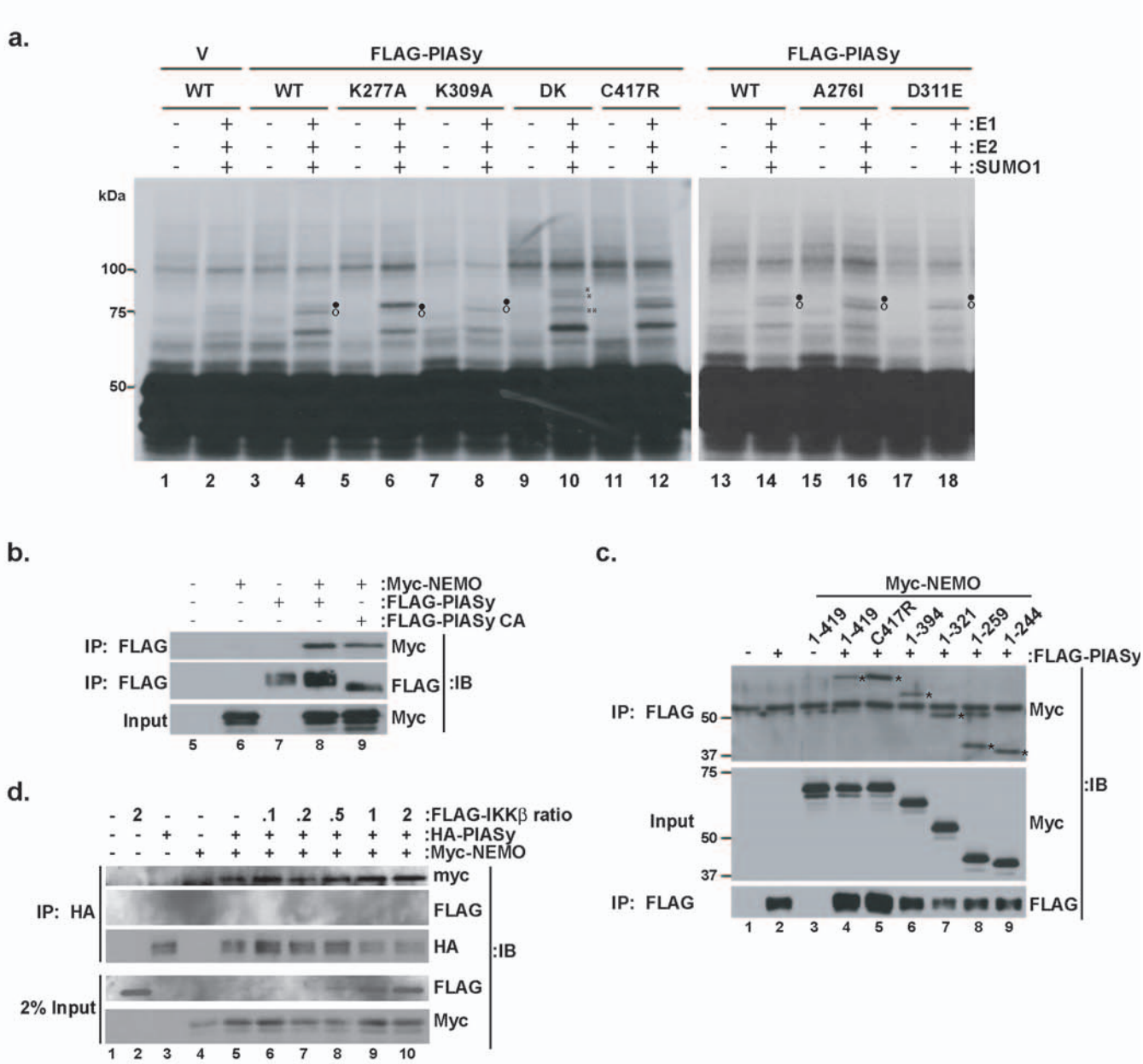

Figure S3: **a.** A similar experiment as shown in Figure 3g was performed using immunopurified human Flag-PIASy instead of His-xPIASy. The SUMOylation banding patters of different NEMO mutants are nearly indistinguishable from those shown in Figure 3g. **b.** HEK293 cells were transfected with Flag-PIASy, Myc-NEMO, and Flag-PIASy-CA. Cells were harvested and lysed, and cell extracts were immunoprecipitated using anti-Flag antibody. Western blotting was performed using anti-Myc and anti-FLAG antibodies. **c.** HEK293 cells were transfected with Flag-PIASy and Myc-NEMO mutants. Cells were immunoprecipitated with an anti-Flag antibody and probed with an HRP-conjugated anti-Myc antibody. **d.** HEK293 cells were transiently transfected with HA-PIASy, Flag-IKKβ, and Myc-NEMO constructs. Flag-IKKβ was titrated in increasing DNA ratios with PIASy. Cell extracts were immunoprecipitated with an anti-HA antibody and probed with anti-Flag and HRP-conjugated anti-HA and anti-Myc antibodies.

Mabb et al-Supplemental Figure S4

RT-PCR Primers	+ strand	- strand
GAPDH:	5'-GTCTTACTCCTTGGAGGCCATG-3'	5'-ACCCCTTCATTGACCTCAACTAC-3'
Pc2:	5'-GAGAACATCCTGGACCCCAGGC-3'	5'-CTGGGCGCCTCCTTGTGGCCGC-3'
PIAS1:	5'-GAACTCCAAGTACTGTTGGGCT-3'	5'-CGGATGGACTGGGTGAAGAGCT-3'
PIAS3:	5'-GGTGCTTCTTGGCTTTGCTGGC-3'	5'-GCTGGCTAGAAGTGGATGCAAG-3'
PIASxα:	5'-CCAGCCAACCGTGTACAAAAATAG-3'	5'-TTCTTTGTTCTCCTGGCAAATC-3'
PIASxβ:	5'-CCAGCCAACCGTGTACAAAAATAG-3'	5'-CTGGTGGTGGTGACAGACGTAC-3'
PIASy:	5'-CTTTAATATGCTGGATGAGCTG-3'	5'-CTCCTTGACCAGTGCCTTGCAC-3'
AL136/MMS21-like:	5'-TGCAGGTCAGCGTCAATGCCAC-3'	5'-GCACTTCAGGGACACCTTGATA-3'
IL-8:	5'-TGCAGCTCTGTGTGAAGG-3'	5'-CTCAGCCCTCTTCAAAAAC-3'
siRNA Oligonucleotides		
Control:	5'-UACCGUCUCCACUUGAUCGdTdT-3'	5'-CGAUCAAGUGGAGACGGUAdTdT-3'
PIASy oligo #2:	5'-CAAGACAGGUGGAGUUGAUUU-3'	5'-PAUCAACUCCACCUGUCUUGUU-3'
PIASy oligo #4:	5'-AAGCUGCCGUUCUUUAAUAUU-3'	5'-PUAUUAAAGAACGGCAGCUUUU-3'
Real Time RT-PCR Primers		
GAPDH:	5'-GAAGGTCGG-AGTCAACGGATTT-3'	5'-GAATTTGCCATGGGTGGAAT-3'
IL-8:	5'-GCAGCTCTGTGTGAAGGTGC-3'	5'-CGCAGTGTGGTCCACTCTCA-3'
IκBα:	5'-GCTACCAACTACAATGGCCACA-3'	5'-TAGCCATGGATAGAGGCTAAGTGTAGA-3'
p21:	5'-GCAGACCAGCATGACAGATTTC-3'	5'-GCGGATTAGGGCTTCCTCTT-3'

Figure S4: Sequences of RT-PCR primers and some of the siRNA oligos used in this study.

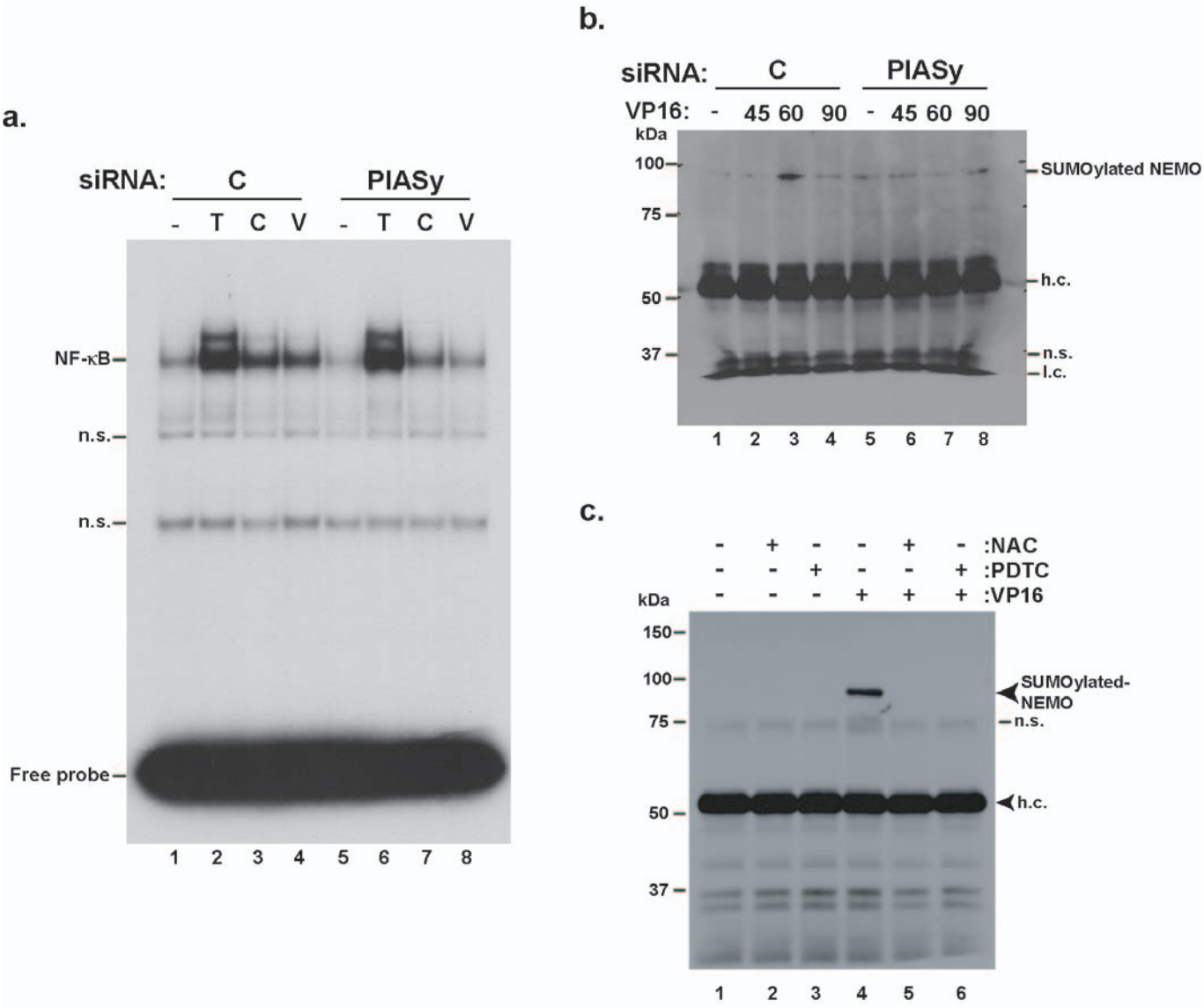

Figure S5: Full scans of original EMSA gel and Western blots. **a.** Full scan of the top panel of Figure 1b EMSA gel. n.s. refers to a nonspecific band, **b.** Full scan of the top panel of Figure 2a SUMO-1 Western blot. h.c. refers to IgG heavy chain and l.c. refers to light chain. **c.** Full scan of the top panel of Figure 5a SUMO-1 Western blot.

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Research article

SUMO and NF-κB ties

A. M. Mabb and S. Miyamoto*

Program in Molecular and Cellular Pharmacology, Department of Pharmacology, University of Wisconsin, 301 Medical Sciences Center, 1300 University Avenue, Madison, WI 53706 (USA), Fax: +1 608 262 1257, e-mail: smiyamot@wisc.edu

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Abstract. Members of the NF-κB family of transcription factors play critical roles in regulating immunity and cell survival and contribute to cancer progression and chemoresistance. Over the past 20 years, much has been learned about the remarkable complexity in regulation of NF-κB signaling. In particular, recent studies have added to our current understanding of the roles of a multitude of post-translational modifications in this signaling system: these include phosphorylation, acetylation, nitrosylation, ubiquitination, neddylation and sumoylation. This review will highlight our current knowledge of the roles of sumoylation in regulating NF-κB signaling and functions and will address future perspectives.

Keywords. SUMO, NF-kappaB, posttranslational, transcription, Ubc9, PIAS, NEMO, IkappaBalpha.

Overview of the NF-κB signaling system

It has been more than 20 years since the initial report of the discovery of the transcription factor nuclear factor kappa B (NF-κB) [1]. NF-κB refers to a collection of protein dimers, each composed of members of the NF-κB/Rel family of proteins. In the mammalian system, there are five members: p65/RelA, RelB, c-Rel, p105/p50 and p100/p52. Conserved members of this family can also be found in other eukaryotic systems, including Drosophila (Dorsal, Dif and Relish), cnidarians (Nv-NF-κB) and others [2]. In mammalian cells, the most ubiquitous NF-κB dimer consists of a p65/p50 heterodimer [3] that is found in the cytoplasm bound to members of a family of inhibitors known as inhibitor of NF-κB (IκB). The binding of IκB family members, such as IκBα and IκBβ, can prevent nuclear accumulation of NF-κB, in part *via* masking the nuclear localization sequence (NLS) of NF-κB [4, 5]. IκBα can also promote nuclear export of bound NF-κB, further ensuring cytoplasmic localization of inactive NF-κB dimers [6–10].

Activation of NF-κB involves its release from IκB, followed by nuclear translocation and a multitude of posttranslational modifications that enable its transcriptional activation function. In this review, the term "signaling" (or "signal transduction") is used specifically to refer to molecular events leading up to, but not including, those directly associated with NF-κB-dependent transcription in the nucleus. The "canonical" NF-κB signaling includes the release of active NF-κB in the cytoplasm by the activation of the cytoplasmic kinase complex, known as the IκB kinase (IKK). IKK consists of three major proteins: two kinases, IKKα/IKK1 and IKKβ/IKK2, and a regulatory subunit, IKKγ/NEMO (NF-κB essential modulator). A large body of literature describes the complex mechanisms that are employed to cause IKK activation in response to a wide array of structurally and functionally unrelated extracellular and intracellular signals [11]. In most cases, activated

* Corresponding author.

IKK promotes phosphorylation of IκBα and its subsequent degradation by the ubiquitin-dependent 26S proteasome system, thereby exposing the NLS of NF-κB, leading to its translocation into the nucleus. During its liberation from IκBα and subsequent to nuclear translocation, NF-κB subunits can undergo several posttranslational modifications to acquire maximal capacity to regulate the transcription of many different target genes involved in inflammatory responses, B and T cell receptor activation and various stress responses. The specific outcomes of NF-κB-dependent transcription are dependent on the cell types and the signals leading to NF-κB activation [12]. In addition to this highly studied NF-κB activation mechanism, another well-established "noncanonical" pathway has also emerged, which involves proteolytic processing of p100/NF-κB2 to selectively activate p52/RelB heterodimers. This pathway is solely dependent on IKKα, without the need for IKKβ and NEMO, and regulates secondary lymphoid organogenesis and B cell development, among other biological functions [13]. Readers are encouraged to consult more comprehensive and excellent reviews of the NF-κB family members, distinct activation mechanisms and their role in transcription [2, 3, 12].

It has been demonstrated time and again that differential posttranslational modifications aid in determining the specificity of signal transduction pathways both activating and sometimes inhibiting NF-κB induced by diverse agents. Modifications such as phosphorylation, ubiquitination and acetylation and, more recently, nitrosylation, neddylation and sumoylation have been demonstrated to play critical roles in regulating NF-κB signaling and transcriptional function [14]. One area of NF-κB biology that has not been extensively reviewed in the literature is the role of protein modifications by SUMO (small ubiquitin-like modifier). Thus, the focus of this review will be geared towards current knowledge regarding the roles of sumoylation of different protein targets in NF-κB signaling and transcriptional pathways. We will first briefly describe the process of sumoylation and then delve into different situations in which sumoylation and SUMO regulatory proteins have been implicated in the regulation of NF-κB. Finally, we will highlight some of the unanswered issues that may be further addressed in future research.

Overview of protein sumoylation

Sumoylation is the process of posttranslational covalent modification of target proteins by a relatively small peptide (~20 kDa) called SUMO. SUMO was cloned over 10 years ago in *Saccharomyces cerevisiae* and called *SMT3* (suppressor of mif two 3) [15]. In humans there are currently four known SUMO isoforms (SUMO-1, SUMO-2, SUMO-3 and SUMO-4), of which only SUMO-1, -2, and -3 can be conjugated to target substrate proteins [16]. SUMO is first translated in an unconjugatable precursor state and needs to be C-terminally processed by SUMO proteases to expose glycine-glycine ("di-glycine") residues critical for the conjugation reaction (see below). SUMO-4 contains a proline residue N-terminally adjacent to the di-glycine moiety, which seems to prevent its maturation into a functional conjugatable entity [17]. Protein modification by SUMO occurs on specific lysine residues of target proteins and takes place *via* a series of enzymatic steps. A heterodimeric SUMO-activating enzyme (E1), SAE1/SAE2 in human (also known as Aos1/Uba2 in *S. cerevisiae*), forms a high-energy thioester bond with the active site cysteine residue C173 in SAE2 and the C-terminal glycine residue of SUMO in a manner that is ATP-dependent. The SUMO moiety is then transferred to the catalytic cysteine residue (C93) of Ubc9 (ubiquitin-conjugating enzyme 9), the SUMO-conjugating enzyme (E2). The E2 can then directly interact with a substrate by recognition of a consensus sequence, ψKxE/D ("ψ" representing a hydrophobic amino acid residue and "x" representing any amino acid residue), and transfer SUMO to the epsilon amino group of the lysine residue embedded within the consensus site to form a covalent isopeptide bond; this is referred to as sumoylation [18]. In yeast and mammals, there is only one known SUMO E1 and one known SUMO E2. Both SUMO E1 and E2 are predominantly found in the nuclear compartment, although the E2 has also been shown to be localized to the cytoplasmic filament of the nuclear pore complex (NPC) [19]. Sumoylation affects many protein substrates and is involved in many important physiological and pathological processes [16, 20]. In fact, deletion of Ubc9 has been shown to be lethal in both yeast and mice [21, 22].

Similar to the ubiquitination system [23], sumoylation can utilize a group of enzymes known as SUMO ligases (E3), which can promote the protein sumoylation reaction both *in vitro* and *in vivo*. There are multiple SUMO E3s identified, and the list is still growing. Like E1 and E2, the cellular location of E3s appears to be concentrated in the nucleus or on the nuclear membrane [24]. The largest group of SUMO E3s belongs to the protein inhibitors of activated STAT (PIAS) family, which was originally identified to be inhibitors of the STAT (signal transducer and activator of transcription) family of transcription factors [25, 26]. Characteristic of the PIAS family is their SP-RING (Siz/PIAS-really interesting new gene) domain, which is critical to promote protein

sumoylation [27]. Two additional SUMO E3s, RanBP2 (Ran-binding protein 2) and Pc2 (polycomb group (PcG) protein), have been identified; however, they are structurally dissimilar to the PIAS family as well as to each other [28, 29]. It has been proposed that SUMO E3s may orient the SUMO-bound E2 onto the protein target to increase sumoylation efficiency [30]. SUMO E3s can also provide some substrate specificity; however, the specificity does not appear to be too strict, as certain SUMO E3s exhibit an ability to promote sumoylation of multiple seemingly unrelated substrates both *in vitro* and *in vivo* [24, 31]. Given the relatively small number of E3s currently known compared to the large number of SUMO substrates identified, it seems logical that many substrates share specific E3s or may not even require the aid of an E3 for efficient sumoylation *in vivo*.

Protein sumoylation is a highly dynamic process in the cell, and the detection of sumoylated proteins has proven very difficult with many protein substrates *in vivo* [16]. Limited pools of SUMO precursors and mature SUMO isoforms and the existence of a family of SUMO-specific proteases (SENPs) are most probably the major contributors to the dynamic nature of sumoylation [32]. The SENPs are a group of cysteine proteases that not only participate in maturation of SUMO precursors (often referred to as "processing") but also cleave isopeptide bonds of SUMO-conjugated protein targets (referred to as "desumoylation") [33]. The first SENP was also discovered in yeast and named Ulp (Ubl-specific protease) 1 [34]. Currently, there are 6 human SENPs known (SENP1, 2, 3, 5, 6 and 7). SENP1 is localized in the nucleus [35, 36], and SENP2 is associated with the nuclear envelope [37, 38]. Both SENP3 [39] and SENP5 [40, 41] localize to the nucleolus, and SENP6 [42, 43] and SENP7 [44] also have nuclear distribution. It seems clear that SUMO proteases vary in their ability to both process different SUMO precursors and differentially desumoylate different SUMO isoforms from distinct target substrates [40, 41, 43, 45–47]. Defining substrate and SUMO isoform deconjugation specificities for each of the SUMO proteases is an important current topic of study. In addition, knockout mouse strains missing one of the SUMO E3s and proteases have been generated to provide insights into their physiological roles [48–51]. However, these studies are incomplete at present.

Sumoylation components regulating NF-κB signaling/transcriptional activity

Case 1: The role of IκBα sumoylation

The first SUMO-modified protein implicated in NF-κB regulation is IκBα [52]. This particular NF-κB inhibitor plays multiple critical roles in regulating both initial activation of NF-κB and the duration of this activity in response to extracellular signals. As described below, sumoylation of IκBα could participate at these different steps (Fig. 1). First, degradation of IκBα in response to various cellular stimuli results in initial NF-κB activation [53]. The degradation of IκBα occurs through phosphorylation on serines 32 and 36, which then leads to its recognition by an SCF (Skp1/Cul1/F-box protein) ubiquitin ligase containing β-TrCP (beta-transducin repeat-containing protein). This in turn causes the K48-linked "canonical" polyubiquitination of IκBα predominantly on K21 and/or K22 and results in its proteasome-mediated degradation [54–57]. The SUMO E2 Ubc9 was first shown to interact with IκBα *in vitro*, and overexpression of a catalytically inactive mutant of Ubc9 delayed both IκBα degradation and NF-κB activation induced by TNFα (tumor necrosis factor-alpha) stimulation [58]. In an independent line of investigation, a SUMO-1-modified form of IκBα that was resistant to ubiquitin-mediated degradation was detected in several cell lines [52]. Ubc9 was further shown to function as a SUMO E2 for IκBα. The resistance of sumoylated IκBα to ubiquitin-dependent degradation was found to be the result of targeting of the same lysine residue normally used for ubiquitination, namely K21 [52] . This landmark study demonstrated that competition of sumoylation and ubiquitination for the same lysine can lead to inhibition of proteasomal degradation of a specific protein substrate.

Previous studies demonstrated that ubiquitination of IκBα does not absolutely depend on K21 and can take place on K22 alone to promote proteasome-mediated degradation [56]. It is possible that due to its globular structure, sumoylation of K21 hinders ubiquitination of K22 by the large β-TrCP SCF complex. Since the N-terminal domain of IκBα appears to be quite flexible [4, 5], K21 sumoylation might also induce a conformational change to prevent recognition by the SCF complex. Interestingly, mutation of the IKK phosphorylation sites S32 and S36 to glutamic acid prevented IκBα sumoylation *in vitro* [52]. Thus, a conformational change could also be associated with modulation of IκBα sumoylation by IKK-dependent phosphorylation.

Surprisingly, later studies revealed that amino acids 1–26 of IκBα could be SUMO-modified *in vitro* but

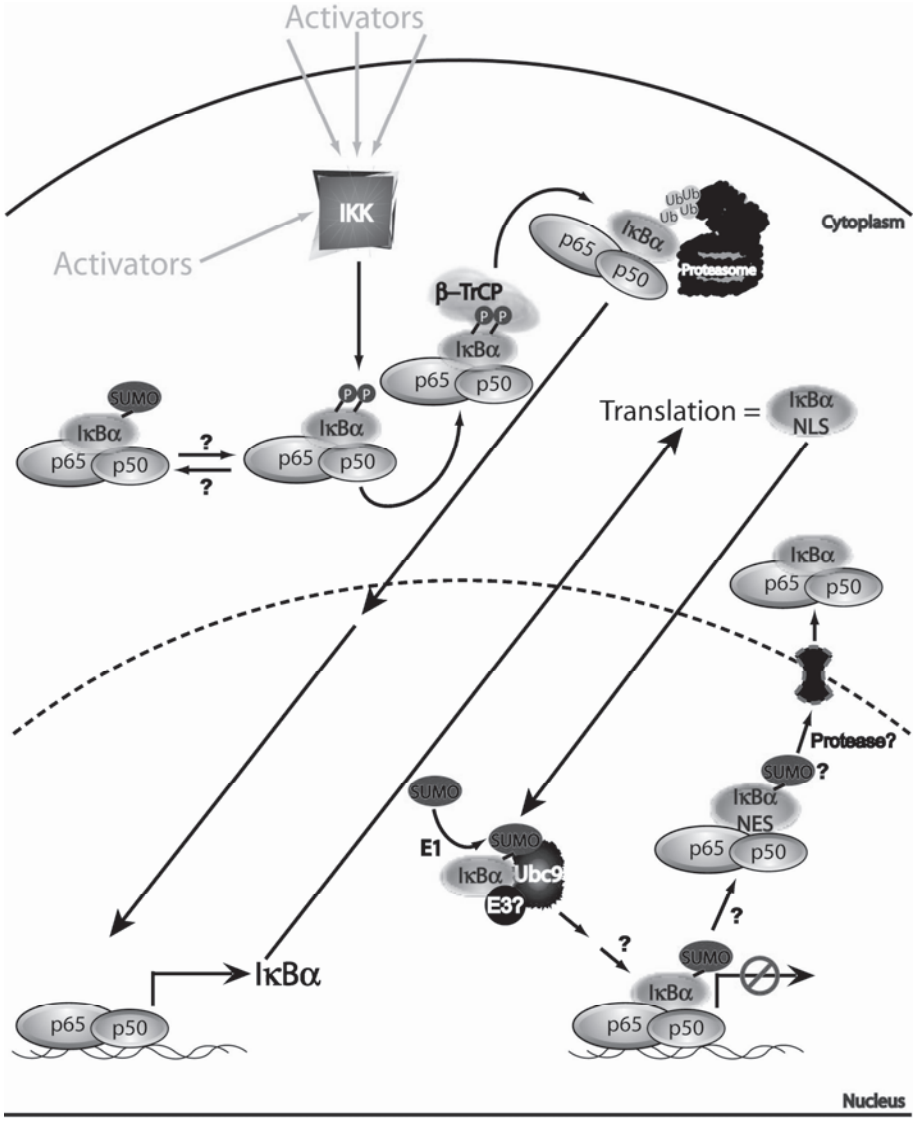

Figure 1. Model showing alternative ways in which sumoylated IκBα could affect the activity of NF-κB. Activators of NF-κB lead to IKK-mediated phosphorylation of IκBα on S32 and S36. This recruits the ubiquitin ligase β-TrCP and results in polyubiquitination of IκBα on K21 and/or K22, leading to its degradation *via* the 26S proteasome. NF-κB then translocates into the nucleus and promotes transcription of its target genes, such as IκBα. Newly translated free IκBα can enter the nucleus *via* its nuclear localization sequence (NLS) and bind NF-κB to inhibit its transcriptional activity. IκBα then brings NF-κB into the cytoplasm *via* its nuclear export sequence (NES), resulting in a cytoplasmic inactive NF-κB/IκBα complex. Sumoylation on K21 has been implicated in inhibition of IκBα degradation, thus providing a mode of NF-κB inhibition. IκBα requires a nuclear signal for its sumoylation that may or may not be enhanced by a SUMO E3. Sumoylated IκBα could play a role in modulating NF-κB-dependent transcription in the nucleus and/or the export of NF-κB from the nucleus. The SUMO protease for IκBα remains unknown. Since most SUMO proteases are localized in the nucleus or at the nuclear periphery, it is possible that an unknown SUMO protease could desumoylate IκBα in conjunction with its exit from the nucleus.

not *in vivo* [59]. Addition of an NLS to IκBα could restore its sumoylation *in vivo*, suggesting that nuclear localization of IκBα was necessary for its sumoylation. Interestingly, free IκBα has been demonstrated to actively transport to the nucleus *via* a nonclassical nuclear localization sequence (NLS) [60]; this nonclassical NLS is located within its second ankyrin repeat and consists of a small cluster of hydrophobic residues [61]. Nuclear localization of free IκBα has an important function in the autoregulatory feedback of NF-κB activity. In a simple yet elegant mechanism, active NF-κB promotes the transcription of IκBα due to an NF-κB responsive element in its promoter [62, 63]. Newly translated IκBα enters the nucleus *via* its unusual NLS and can then "strip" NF-κB off of its DNA element [64, 65] and bring it back to the cytoplasm *via* an IκBα nuclear export sequence (NES). Two NES motifs in IκBα have been identified, one located on its N-terminus and one with a C-terminal location [6–10]. Cell-based analyses support a critical role for both of these NES sequences in the regulation of NF-κB; however, mouse knock-in studies might be necessary to determine the physiological functions of the two sequences. Whether sumoylation of IκBα participates in this elaborate "post-induction repression" process has not been directly evaluated. As sumoylation of IκBα requires its nuclear localization, this may be the case, as previously proposed by others [59, 66]. In this context, the modulatory effects of sumoylation of IκBα on its affinity for NF-κB, subcellular localization, ability to remove NF-κB off DNA sites and nuclear export of NF-κB have not been carefully analyzed. Furthermore, whether or not a SUMO E3 is involved in sumoylation of IκBα and if there is a specific SUMO protease that removes the SUMO moiety from IκBα

have yet to be determined. These additional factors may also change the balance between sumoylated and non-sumoylated pools of IκBα to further fine-tune NF-κB functions.

It was recently described that overexpression of SUMO-4 could modify IκBα and inhibit NF-κB activation in response to TNFα [67]. However, it has yet to be determined if endogenous proteins can be modified by endogenous SUMO-4; the presence of a proline residue near the di-glycine moiety may prevent maturation of SUMO-4 by SUMO proteases [17].

Case 2: Sumoylation of NEMO during NF-κB signaling induced by DNA-damaging agents

The most recently identified SUMO-modified protein substrate in the NF-κB signaling pathway is NEMO [68] (Fig. 2). NEMO, as stated previously, is part of the cytoplasmic IKK complex that is critical for NF-κB activation not only by the majority of extracellular signals, including TNFα and IL-1 (Interleukin-1), but also in response to many genotoxic stress agents, such as etoposide (VP16), camptothecin (CPT) and ionizing radiation (IR) [53, 69]. Structurally, from its N- to C-terminus, NEMO is predicted to contain two putative coiled coil domains (CC1 and CC2), a leucine zipper (LZ) and a zinc finger domain (ZF). NEMO is capable of multimerization; however, the stoichiometry of this multimer appears to be complex *in vivo*, given that dimeric, trimeric and tetrameric forms of NEMO have been described [70–73].

Multimerization of NEMO has been proposed to be critical for activation of the IKK complex, and its multimerization is thought to require the CC2 and LZ trimerization domain on one hand [72] and the N-terminal dimerization domain on the other [73]. Moreover, forced dimerization of NEMO is sufficient to cause IKK and NF-κB activation [70].

The uniqueness of the role of SUMO in genotoxic stress signaling to NF-κB lies upstream of IKK activation events. It was previously demonstrated that enucleation had little impact on NF-κB signaling induced by TNFα, thereby indicating that all the signaling components and events can take place without the need for an intact nucleus. In contrast, the enucleation procedure abrogated activation by CPT, a topoisomerase I poison [74]. This study suggested that a nuclear event, or at least an intact nucleus, is selectively required for genotoxic stress-induced NF-κB activation. Since enucleation leaves mitochondria in the cytoplasts [75], and this abundant organelle contains its own DNA that can also serve as a target for CPT to cause DNA damage *via* the mitochondria-specific topoisomerase I enzyme [76], the study demonstrated that nuclear topoisomerase I is the critical target for the NF-κB activation event to take place. Indeed, CEM/C2 cells that harbor intact mitochondrial topoisomerase I but contain a CPT-resistant nuclear enzyme were defective for NF-κB activation by CPT but not by TNFα or other DNA-damaging agents that do not target topoisomerase I [74]. These and additional studies [77] seem to point to DNA double-strand breaks (DSBs) as the critical

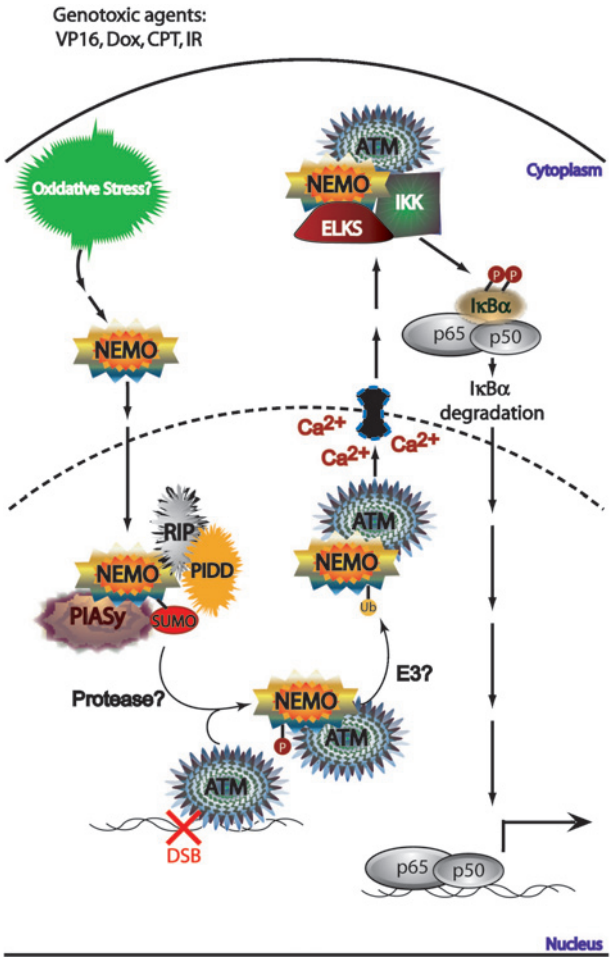

Figure 2. Model depicting the role of sumoylation in genotoxic stress-induced NF-κB signaling. Genotoxic agents cause DNA double-strand breaks (DSBs) along with other cellular stress, including oxidative stress. The latter might be involved in modulating NEMO localization to the nucleus, possibly *via* the C-terminal ZF domain. NEMO is then SUMO-modified on K277/K309 with the help of the SUMO E1/E2 and an E3, PIASy. PIDD and RIP-1 also have a role in NEMO sumoylation. DSBs lead to the activation of ATM, which then associates with NEMO and promotes phosphorylation on S85. This in turn results in NEMO ubiquitination, supposedly on the same lysines that are used for sumoylation. NEMO is exported out of the nucleus likely with ATM, a mechanism that requires Ran-GTP, an unknown nuclear export receptor and Ca^{2+}. Exported ATM and NEMO then work with a protein known as ELKS to promote IKK activation, resulting in activation of NF-κB *via* IκBα degradation. The nature of a SUMO protease, a ubiquitin ligase (E3) and the nuclear export receptor for NEMO and the mechanism of IKK activation remain undetermined.

DNA lesion in promoting NF-κB activation by many genotoxic agents. Later, ataxia telangiectasia mutated (ATM), a nuclear DSB-activated protein kinase, was shown to be essential for NF-κB activation in response to DSB inducers [68, 78]. Although these findings and others [79–81] suggest the requirement for a nuclear signaling event for genotoxic stress-induced NF-κB activation, they did not explain how ATM signaling caused cytoplasmic IKK activation and subsequent NF-κB activation.

A surprising series of events led to a partial understanding of the relationship between NEMO sumoylation and ATM-dependent activation of the IKK complex leading to NF-κB activation. It was demonstrated that a fraction of IKK-free NEMO was SUMO-1-modified in response to CPT and VP16 but not by TNFα and LPS (lipopolysaccharide), and this caused its nuclear accumulation in the cell [68]. This sumoylation was specific for SUMO-1 (not SUMO-2 or -3), since SUMO-2- or SUMO-3-modified NEMO was not observed [82]. SUMO-1 modification of NEMO was induced in a transient manner that occurred prior to cytoplasmic IKK and NF-κB activation. ATM was found to be irrelevant for promoting sumoylation of NEMO by DSB-inducing agents [68]. Moreover, this sumoylation could also be induced by cell stresses that did not induce DSBs or ATM activation [83], thereby further implicating non-ATM-mediated, but some stress-mediated, events in NEMO sumoylation. The putative SUMO-modification sites were determined by mutagenesis studies to be K277 and K309 within and just outside the CC2 domain, respectively [68]. The sumoylation of NEMO also required its ZF motif *in vivo*, and a ZF mutant of NEMO was incapable of being SUMO-modified and could not accumulate in the nucleus upon CPT and VP16 exposure. Following sumoylation, nuclear NEMO interacted with ATM, which phosphorylated serine 85 of NEMO to promote its ubiquitin modification and nuclear export [84]. As mentioned with IκBα above, the sumoylation and ubiquitination sites appeared to compete for the same lysine sites. Mutation of both lysines to alanine prevented ubiquitination even when SUMO-1 was supplied to NEMO as a fusion moiety, a trick that could complement the ZF-dependent sumoylation defect of NEMO [68]. A recent study further demonstrated that a Ca^{2+}-mediated signaling event is also required for NEMO export into the cytoplasm [85]. Cumulation of data led to the model that nuclear export of NEMO permits ATM to form a complex with cytoplasmic IKK, leading to IKK and NF-κB activation by an as-yet poorly defined mechanism [84]. Inhibition of any of these events eliminated genotoxic stress-induced NF-κB activation. Gene fusion studies indicate that SUMO-1 or ubiquitin directly fused to NEMO can bypass the need for these modifications [68, 84], further supporting the critical roles of these events.

The sumoylation of NEMO is believed to be independent of IKK complex formation. Evidence that supports this model include that dissociation of NEMO from the IKK complex by a NEMO-binding peptide did not disrupt its sumoylation. In contrast to NEMO, nuclear translocation of the IKK subunits (IKKα and IKKβ) was not observed prior to NF-κB activation [68]. This suggests that there is a preexisting IKK-free pool of NEMO or some fraction of NEMO that is dissociated from the IKK complex following stress induction to allow for SUMO modification. The requirement of IKK-free NEMO for sumoylation became evident when the SUMO E3 for NEMO was identified. At the time of the study, the known SUMO E3s consisted of the proteins Pc2 and RanBP2, two unrelated SUMO ligases and the PIAS family consisting of PIAS1, PIAS3, PIASxα, PIASxβ and PIASy. Utilizing siRNA towards these known SUMO E3s, a PIAS family SUMO E3, PIASy (also known as PIASγ), was shown to be specifically required for NF-κB activation in response to genotoxic stress [82]. PIASy was demonstrated to promote NEMO sumoylation both *in vitro* and *in vivo* in a manner that was dependent on its catalytic activity (SP-RING domain), and it was also shown to be necessary for NEMO sumoylation in response to genotoxic stimuli without interfering with ATM activation [82]. Significantly, PIASy was only able to interact with IKK-free NEMO. Even when IKKβ and PIASy were overexpressed, NEMO was unable to assemble an IKKβ-NEMO-PIASy complex, demonstrating that the interaction between NEMO-PIASy and NEMO-IKKβ was mutually exclusive; indeed, an overlapping domain of NEMO was required for these interactions. Moreover, the NEMO-PIASy interaction appears to be predominantly nuclear. Interestingly, the NEMO ZF domain was dispensable for both interaction with PIASy *in vivo* and direct SUMO-1 modification *in vitro*. Additionally, sumoylation of NEMO by Ubc9 and PIASy was selective for SUMO-1, since SUMO-2 and SUMO-3 were not conjugated to NEMO *in vitro*. Certain antioxidants prevented NEMO sumoylation induced by VP16 in CEM T leukemic cells [82] and oxidative stress by means of exogenously added hydrogen peroxide was able to cause NEMO sumoylation. These findings led to a model in which cell stress, possibly an oxidative stress-related event, promotes ZF-dependent nuclear import of NEMO and subsequent PIASy-dependent SUMO-1 modification without involving ATM activation.

A recent study further contributed to our understanding of the control of NEMO sumoylation by genotoxic stress conditions. Janssens et al. found that the protein PIDD (p53-inducible death-domain-containing protein) translocates to the nucleus to stabilize/enhance NEMO sumoylation in the cell following genotoxic insult [86]. The increase in SUMO-modified NEMO also correlated with augmented NF-κB activation. It was further shown that RIP1 (receptor-interacting protein 1), previously found to be critical for genotoxic stress-induced NF-κB activation [87], was also required for NEMO sumoylation in conjunction with PIDD. siRNA-mediated silencing of endogenous PIDD attenuated NEMO sumoylation and NF-κB activation, indicating that PIDD is critical for efficient NF-κB activation by genotoxic agents (see Fig. 2 for model). A more recent study demonstrated that PIDD undergoes autocleavage processing to generate PIDD-C and PIDD-CC peptides [88]. Interestingly, PIDD-C promoted NF-κB activation by genotoxic agents, whereas PIDD-CC specifically assembled with RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain) and procaspase-2 to cause apoptosis [88]. It is still unclear how PIDD-C promotes NEMO sumoylation; however, these additional molecular components add to the exciting, yet complex nature of the NEMO sumoylation regulation induced by DNA-damaging agents.

Of note, other roles for PIASy in regulating NF-κB function have recently been observed. Overexpression of PIASy inhibited NF-κB-dependent luciferase reporter activity in both a 291D-GFP mouse keratinocyte cell line in response to a combination of UVB (ultraviolet B) and TNFα in one study [89] and in HEK293 cells in response to overexpressed TRIF (Toll/IL-1 receptor (TIR) domain-containing adaptor inducing IFN-β) [90]. Whether or not PIASy is acting in these pathways through NEMO sumoylation or through an unidentified NF-κB signaling protein has yet to be determined. The requirement of the catalytic activity of PIASy to mediate the inhibitory action has not been determined in these studies, so it also remains a possibility that the effect of PIASy may be mediated by protein-protein interactions without the need for sumoylation *per se*.

Case 3: SUMO, SUMO enzymes and NF-κB-dependent transcription

A. NF-κB transcriptional repression. An instance in which sumoylation components affect NF-κB transactivation activity was shown with a SUMO E3 PIAS family member, PIAS1. In the first scenario, PIAS1 was shown to interact with the NF-κB family member p65 (RelA), and overexpression of PIAS1 led to inhibition of NF-κB-dependent transcription, as shown using an NF-κB luciferase reporter construct [91]. It was further demonstrated that PIAS1 could also inhibit the NF-κB-dependent target genes *IκBα* and *Bfl1*. PIAS1 was believed to exert its effects through prevention of NF-κB DNA-binding activity in the nucleus of the cell, since PIAS1 inhibition of NF-κB occurred after IκBα degradation and p65 nuclear translocation. Overexpression of PIAS1 could inhibit DNA binding of NF-κB *in vitro* and on the *IκBα* promoter *in vivo*. In PIAS1$^{-/-}$ mice, transcription of the NF-κB-dependent genes for the proinflammatory cytokines TNFα and IL-1β was increased upon stimulation with TNFα. These mice also exhibited slightly elevated basal serum levels of TNFα and IL-1β, indicating that PIAS1 functions to suppress NF-κB-dependent transcription [91]. Furthermore, using chromatin immunoprecipitation (ChIP) assays in a PIAS1-deficient cell line, an increase in p65 recruitment to the *IκBα* promoter was seen upon activation of NF-κB with TNFα. Hence, the ability of PIAS1 to prevent NF-κB-dependent transcription *in vivo* correlated with inhibition or displacement of p65 occupancy from the promoter site. Interestingly, PIAS1 was shown to interact with the transactivation domain (TAD) of p65. Thus, in this case, the inhibitory action of PIAS1 on NF-κB-dependent transcription appears to involve direct protein-protein interactions rather than PIAS1-mediated sumoylation of p65.

In the second setting, PIAS1 also surfaced as a regulator of NF-κB-dependent transcription *via* modulating sumoylation of a different transcription factor, namely PPAR-γ (peroxisome proliferator-activated receptor gamma) [92]. PPAR-γ is a nuclear receptor with a role in regulation of adipogenesis [93]. PPAR-γ also has anti-inflammatory properties that are believed to be in part due to its ability to inhibit NF-κB-dependent transcription [94]. A recent study examining the effect of corepressor complexes for NF-κB revealed that NCoR (nuclear hormone receptor corepressor) and its related factor SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) in complex with HDAC3 (histone deacetylase 3) can serve as a corepressor complex for a subset of NF-κB-dependent target genes, such as the proinflammatory response gene *iNOS* (inducible nitric oxide synthase) [92]. In a yeast 2-hybrid screen, PIAS1 was identified as a binding partner of PPAR-γ. siRNA targeting PIAS1 demonstrated its requirement for LPS-induced PPAR-γ recruitment to the *iNOS* promoter. Treatment with LPS could also promote PPAR-γ sumoylation, and this was required for transrepression of the *iNOS* promoter. These results led to a model in which LPS-induced sumoylation of PPAR-γ mediated by PIAS1 results in PPAR-γ-

dependent recruitment to the NCoR/SMRT corepressor complex on the *iNOS* promoter. Recruitment of this corepressor prevents NF-κB-dependent transactivation. This PIAS1-PPAR-γ-dependent repression of NF-κB target gene transcription might also play a role in the previous report of PIAS1-mediated NF-κB repression [91]. Analysis of this mechanism on multiple promoters of NF-κB-dependent genes would determine how frequently this mechanism is utilized in the cell.

In a separate study, another SUMO E3, PIAS3, was shown to interact with p65 in a yeast 2-hybrid assay, and this interaction was subsequently confirmed using both *in vitro* and *in vivo* assays [95]. In contrast to PIAS1, PIAS3 interacted with the Rel homology domain (RHD) of p65, the domain responsible for NF-κB dimerization and DNA binding. Furthermore, overexpression of PIAS3 could inhibit TNFα- and IL-1β-induced NF-κB-dependent transcription (shown using a 3xκB luciferase reporter), similar to what was seen with PIAS1. Like PIAS1, the effects of PIAS3 were downstream of IκBα degradation, since overexpression of PIAS3 did not alter phosphorylation-dependent degradation of IκBα in response to TNFα stimulation. Unlike PIAS1, PIAS3 did not seem to alter p65 DNA-binding activity. It is well-established that CBP [CREB (cAMP response element-binding)-binding protein] acts as a coactivator of p65 [96, 97]. Interestingly, when overexpressed, PIAS3 could compete with CBP for binding to p65 both *in vitro* and *in vivo* [95]. Hence, the effects of PIAS3 on p65-mediated NF-κB transactivation appear to be due to defects in coactivator binding to p65. Whether or not the catalytic domain within the RING finger of PIAS3, which has been shown to be required for enhanced substrate sumoylation, is required for inhibition of NF-κB transcription has not been determined. This is also the case with PIAS1, described above. Thus, it is still unclear whether the enzymatic activity of PIAS1/3 is required for repression of NF-κB transcriptional activity. Of note, it is of interest that components of the p65 coactivator complex, CBP and p300, are SUMO-modified themselves [98, 99]. SUMO modification of CBP and p300 in this context serves as a negative regulator of transcriptional activity. It is tempting to think that CBP and p300 coactivator regulation by SUMO modification may play a role in negative regulation of NF-κB transcriptional activity. Further studies will need to be conducted to determine if this mechanism indeed exists.

The strong role of sumoylation in NF-κB regulation and possibly NF-κB-dependent transcription was initially identified in genetic studies of innate immunity in the Drosophila system. Mutations in the Drosophila version of the Ubc9 (dUbc9) gene, *lesswright* (*lwr*), were associated with up-regulation of the NF-κB pathway in the fat body, where increased transcriptional activity of Dorsal (dl) and Dif (Dorsal-related immunity factor), members of the Rel/NF-κB family, resulted in constitutive expression of the antimicrobial peptide genes *Drosomycin* and *Cecropin* [100]. Constitutive expression of these genes is abolished in triple (*lwr*, *Dif* and *dl*) mutants [100]. Moreover, the *dUbc9/lwr* had a separate but similar regulatory effect in Drosophila blood cells (hemocytes). *Lwr* mutants exhibited increased proliferation of hemocytes, and some mutant hemocytes showed localization of Dorsal to the nucleus [101]. The overproliferation phenotype could be suppressed in triple (*lwr*, *Dif* and *dl*) mutants [100, 101], but the target genes of Dorsal/Dif causing the overproliferation were not described. Both the fat body- and hemocyte-based phenotypic defects were also rescued by a non-signaling allele of Cactus [100]. These genetic epistatic experiments suggested that dUbc9 either regulates Dorsal and Dif *via* its effects on Cactus or independently regulates Cactus and Dorsal/Dif in the signal transduction pathway. It is possible that direct sumoylation of Dorsal and Dif results in transcriptional repression of target genes such as that of the mammalian transcription factors c-Jun, p53, androgen receptor (AR), Sp3 and Elk-1 (Ets-like kinase 1) [20]. In this context, it is of interest to note that in Cos7 (transformed monkey kidney) cells, overexpression of Ubc9 or SUMO-1 inhibited NF-κB transcriptional activity induced by TNFα, IL-1 and okadaic acid as measured by an NF-κB-dependent HIV-LTR reporter construct [52]. Alternatively, it is also possible that dUbc9 might function in modifying Cactus stability or in regulating Dorsal localization [100]. A previous study indicated that overexpression of catalytically inactive Ubc9 can retard IκBα degradation and NF-κB activation in response to TNFα stimulation in HeLa cells [58].

B. NF-κB transcriptional activation. Although overexpression of dUbc9 was shown to inhibit Dorsal-dependent transcription, as described above, studies examining the effect of dUbc9 on Drosophila development demonstrated the converse [102]. Dorsal was shown to interact with dUbc9 in a yeast 2-hybrid assay, and this interaction was confirmed *in vivo*. Overexpression of Dorsal in cells can result in its nuclear localization by overcoming inhibition imposed by the endogenous pool of the Drosophila homologue of IκB, Cactus. Hence, Cactus needs to be overexpressed along with Dorsal in order to restore Dorsal's cytoplasmic localization. Under conditions in which Dorsal, Cactus and a coactivator (Twist) were coex-

pressed, overexpression of dUbc9 surprisingly resulted in nuclear accumulation of Dorsal and transactivation of a Dorsal-dependent reporter gene. This is inconsistent with the previous study showing that the lesswright mutation causes an increase in nuclear Dorsal accumulation in hemocytes [101]. Coexpression of Dorsal/Twist/Cactus with Drosophila SUMO or the Drosophila SUMO E1, SAE1/2, could also augment the reporter activity, suggesting that components of the SUMO conjugation machinery could stimulate transcription of NF-κB in the fly [102].

Later, Dorsal was shown to be SUMO-modified on K382 in Drosophila S2 cells [103]. Overexpression of dSUMO and dUbc9 could increase Dorsal-dependent reporter activity and expression of the *CecA1* gene. siRNA targeting dUbc9 and dSUMO led to a reduction in *CecA1* gene induction following LPS stimulation of Drosophila S2 cells. Examination of Drosophila first instar larvae that had hypomorphic P-element disruptions in either dUbc9 or dSUMO also revealed defective *CecA1* gene induction upon challenge with LPS. This is in contrast with the study mentioned above, which demonstrated that mutations in lesswright (dUbc9) can increase *CecA1* gene expression in third instar larvae [100]. These apparent contradictory results in Drosophila larvae and cultured cells support the view that individual proteins (or the stoichiometry of proteins in complexes) subject to sumoylation may have different outcomes depending on the cell system used. These observations also underscore the need to characterize how these biochemical differences result in distinct physiological outcomes. The variable effects of SUMO proteins on NF-κB signaling is summarized to emphasize this idea (Table 1).

Overexpression of dSUMO and dUbc9 could activate Dorsal-dependent reporter activity, and mutation of the SUMO modification site on K383 to A or R also increased Dorsal reporter activity, leaving the question as to how sumoylation and mutation of the putative SUMO modification site in Dorsal could lead to the same phenotype. Using two luciferase reporter constructs, one with a single and one with multiple Dorsal binding sites, it was found that mutant Dorsal was only able to increase luciferase activity when multiple binding sites were present. From these results, Bhaskar et al. [103] concluded that the SUMO modification site of *Dorsal* is actually within a synergy control (SC) motif, which has been shown to inhibit transcriptional synergy through recruitment of a synergy attenuator, an SC factor (SCF) [104]. Both SUMO modification and mutagenesis of K383 to A or R presumably prevent the interaction of an unidentified SC factor with DNA-bound Dorsal/Twist, thwarting the attenuation of Dorsal transactivational activity and thus giving rise to the same transcriptional phenotype. This is analogous to the situation in the mammalian system, in which SC motifs have similarity to sumoylation sites; for example, C/EBPalpha and GR (glucocorticoid receptor) can be SUMO-modified within their SC motifs, leading to uncontrolled gene expression [105, 106]. Thus, although sumoylation of transcription factors is generally linked to their transcriptional repression, there are other instances in which SUMO modification of transcription factors can promote their transcriptional activity. Sumoylation of estrogen receptor alpha (ERα) and heat shock factors 1 and 2 (HSF1 and 2) has also been shown to promote their transcription [107–109]; however, unlike the SC factor situations described above, the mechanisms are unclear as to how direct sumoylation of ERα and HSF1/2 could promote their transcriptional activities.

In another yeast 2-hybrid screen, MEKK1 (mitogen-activated protein kinase/ERK kinase kinase-1), a protein positively implicated in NF-κB signaling [110, 111], was found to directly interact with Ubc9 [112]. Further analysis revealed that this interaction requires the catalytic domain and death domain of MEKK1. Overexpression of Ubc9, regardless of its catalytic activity, was shown to promote TNFα-induced NF-κB transcription in an NF-κB-dependent luciferase reporter assay in HeLa cells. This corroborates a previous study showing that a catalytically dead mutant of Ubc9 retards TNFα-induced NF-κB activation [58]. Moreover, when expressed together, Ubc9 and MEKK1 could act in synergy to induce NF-κB activation in response to TNFα stimulation. It was proposed that Ubc9 may be acting as a scaffolding protein with MEKK1 [112]. We also found that depletion of Ubc9 inhibited NF-κB-dependent luciferase reporter activity in response to TNFα in HEK293 cells (Mabb, unpublished observations). In these scenarios, it appears that Ubc9 has some NF-κB signaling function, possibly without involving sumoylation, upstream of NF-κB transcriptional events.

To help sort out the role of SUMO components in NF-κB signaling and modulation of NF-κB-dependent transcription, we attempted to formulate a model as to how sumoylation and SUMO conjugation machinery may play a role in the NF-κB system in response TNFα (Fig. 3). In this hypothetical model, TNFα binds to the TNFR1 (tumor necrosis factor receptor 1) and causes the recruitment of downstream factors, such as TRADD (TNF-R1-associated death domain protein) and RIP1, to the C-terminal DD (death domain) of TNFR1 [113]. Ubc9 may also impinge on the DD of TNFR1 to mediate protein interactions or promote sumoylation of known or as-yet-unidentified additional factors. MEKK1 is also

Table 1. Putative role of SUMO enzymes in the NF-κB pathway.

Cell line/system used	Stimulus	Sumoylation component(s) examined	Experiment	Suggested effects on substrate	Effects on NF-κB	Reference
HeLa	TNFα	Dominant negative Ubc9	Overexpression	Inhibition of IκBα degradation	Inhibition	[58]
HeLa	TNFα	Ubc9 and MEKK1	Overexpression	Interaction with MEKK1	Activation	[112]
HeLa	TNFα	Ubc9	Overexpression	Effects on MEKK1	Activation	[112]
HeLa	TNFα	Dominant negative Ubc9	Overexpression	Effects on MEKK1	Activation	[112]
Cos7	TNFα	SUMO-1	Overexpression	Effects on IκBα sumoylation	Inhibition	[52]
Cos7	IL-1	SUMO-1	Overexpression	Effects on IκBα sumoylation	Inhibition	[52]
Cos7	Okadaic acid	SUMO-1	Overexpression	Effects on IκBα sumoylation	Inhibition	[52]
Cos7	TNFα	Ubc9	Overexpression	Effects on IκBα sumoylation	Inhibition	[52]
Cos7	IL-1	Ubc9	Overexpression	Effects on IκBα sumoylation	Inhibition	[52]
Cos7	Okadaic acid	Ubc9	Overexpression	Effects on IκBα sumoylation	Inhibition	[52]
HEK 293	VP16	Ubc9	Ubc9 siRNA	Effects on NEMO sumoylation	Inhibition	[82]
HEK 293	TNFα	Ubc9	Ubc9 siRNA	Unknown	Inhibition	Mabb, unpublished
HEK 293	TNFα	SUMO-4	Overexpression	Potential IκBα sumoylation	Inhibition	[67]
Drosophila S2	-	Drosophila Ubc9	Overexpression with Dorsal and Twist	Dorsal	Activation	[103]
Drosophila S2	-	Drosophila Ubc9	Overexpression with Dorsal, Cactus, and Twist	Dorsal	Activation	[102]
Drosophila S2	-	Drosophila Ubc9/ SUMO-1	Overexpression with Dorsal	Dorsal	Activation	[103]
Drosophila S2	-	Drosophila Ubc9/ SUMO-1	Overexpression with Dorsal and Twist	Dorsal	Activation	[103]
Drosophila S2	-	Drosophila SUMO	Overexpression with Dorsal, Cactus, and Twist	Dorsal	Activation	[102]
Drosophila S2	-	Drosophila Ubc9/ SUMO-1	Overexpression with Dorsal, Cactus, and Twist	Dorsal	Activation	[102]
Drosophila S2	-	Drosophila Sae1/2 (SUMO E1)	Overexpression with Dorsal, Cactus, and Twist	Dorsal	Activation	[102]
Drosophila S2	-	Drosophila Sae1/2 (SUMO E1)	Overexpression with Dorsal, Cactus, Twist, and Smt3	Dorsal	Activation	[102]
larval hemocytes	-	Drosophila Ubc9	lesswright hypomorphic P-element disruption	Unknown	Dorsal nuclear localization	[101]
larval hemocytes	-	Dominant negative Drosophila Ubc9	Overexpression	Unknown	Dorsal nuclear localization	[101]
HEK293 and CEM	VP16	PIASy	siRNA	NEMO sumoylation	Inhibition	[82] and Mabb, unpublished
HEK293 and CEM	CPT	PIASy	siRNA	NEMO sumoylation	Inhibition	[82] and Mabb, unpublished

Table 1 *(Continued)*

Cell line/system used	Stimulus	Sumoylation component(s) examined	Experiment	Suggested effects on substrate	Effects on NF-κB	Reference
291D-GFP mouse keratinocyte	UVB/ TNFα	PIASy	Overexpression	Unknown	Inhibition	[89]
HEK 293	-	PIASy	Overexpression with TRIF	Unknown	Inhibition	[90]
HEK 293	-	PIASy	Overexpression with TRAF6	Unknown	Inhibition	[90]
MEF	TNFα	PIAS1	PIAS1$^{-/-}$ MEFs	p65	Increase in NF-κB target genes	[91]
HEK 293T and A549	TNFα	PIAS1	Overexpression	p65	Inhibition	[91]
HEK 293T	TNFα	PIAS3	Overexpression	p65	Inhibition	[95]
HEK 293T	IL-1β	PIAS3	Overexpression	p65	Inhibition	[95]
HEK 293T	-	PIAS3	Overexpression with RANK	p65	Inhibition	[95]
HEK 293T	-	PIAS3	Overexpression with TNFR1	p65	Inhibition	[95]
HEK 293T	-	PIAS3	Overexpression with CD30	p65	Inhibition	[95]
HEK 293T	-	PIAS3	Overexpression with TRAF2	p65	Inhibition	[95]
HEK 293T	-	PIAS3	Overexpression with TRAF5	p65	Inhibition	[95]
HEK 293T	-	PIAS3	Overexpression with TRAF6	p65	Inhibition	[95]
MCF-7	TNFα	PIAS3	siRNA	p65	Activation	[95]
HeLa	TNFα	SENP1	Overexpression	Unknown	Inhibition	[117]
HeLa	TNFα	Catalytic defective SENP1	Overexpression	Unknown	Inhibition	[117]
Drosophila S2	-	Drosophila Ulp1	Overexpression with Dorsal and Twist	Dorsal	Biphasic response	[103]

Known SUMO-modified proteins in the NF-κB pathway

Protein	stimulus	Site Required for Modification	Effects of modification on NF-κB signaling	
IκBα	TNFα	K21	Inhibition of IκBα degradation	[52]
NEMO	VP16, CPT	K277/K309	Activation	[68]
NEMO	H2O2	-	Activation	[83]
NEMO	VP16, Dox	-	Activation	[86]
Dorsal	-	K382	Activation?	[103]

activated and interacts with Ubc9 to cause activation of downstream components that promote IKK activation leading to phosphorylation-mediated degradation of IκBα and translocation of NF-κB into the nucleus. Here, Ubc9 may also be involved in the interaction with p50 [58] to either promote or inhibit transcription depending on the cellular context. Upon DNA binding and transcription of NF-κB target genes, PIAS1 may be recruited to a subset of the genes to dissociate NF-κB from its promoter site, perhaps through facilitating SUMO modification and/or displacing bound NF-κB with an unknown inhibitory factor similar to NcoR/SMRT for PIAS1. Alternatively, PIAS3, along with a potential sumoylated corepressor, could be recruited to a subset of NF-κB target genes to prevent recruitment of one of its coactivators, CBP. The signal specificity for PIAS1 or PIAS3 may depend on the context of the promoter. Depending on its positioning inside the cell, the existence of SUMO-modified IκBα may further prevent NF-κB target gene transcription through either sequestration of cytoplasmic NF-κB or sequestration of nuclear NF-κB to prevent target gene transcription.

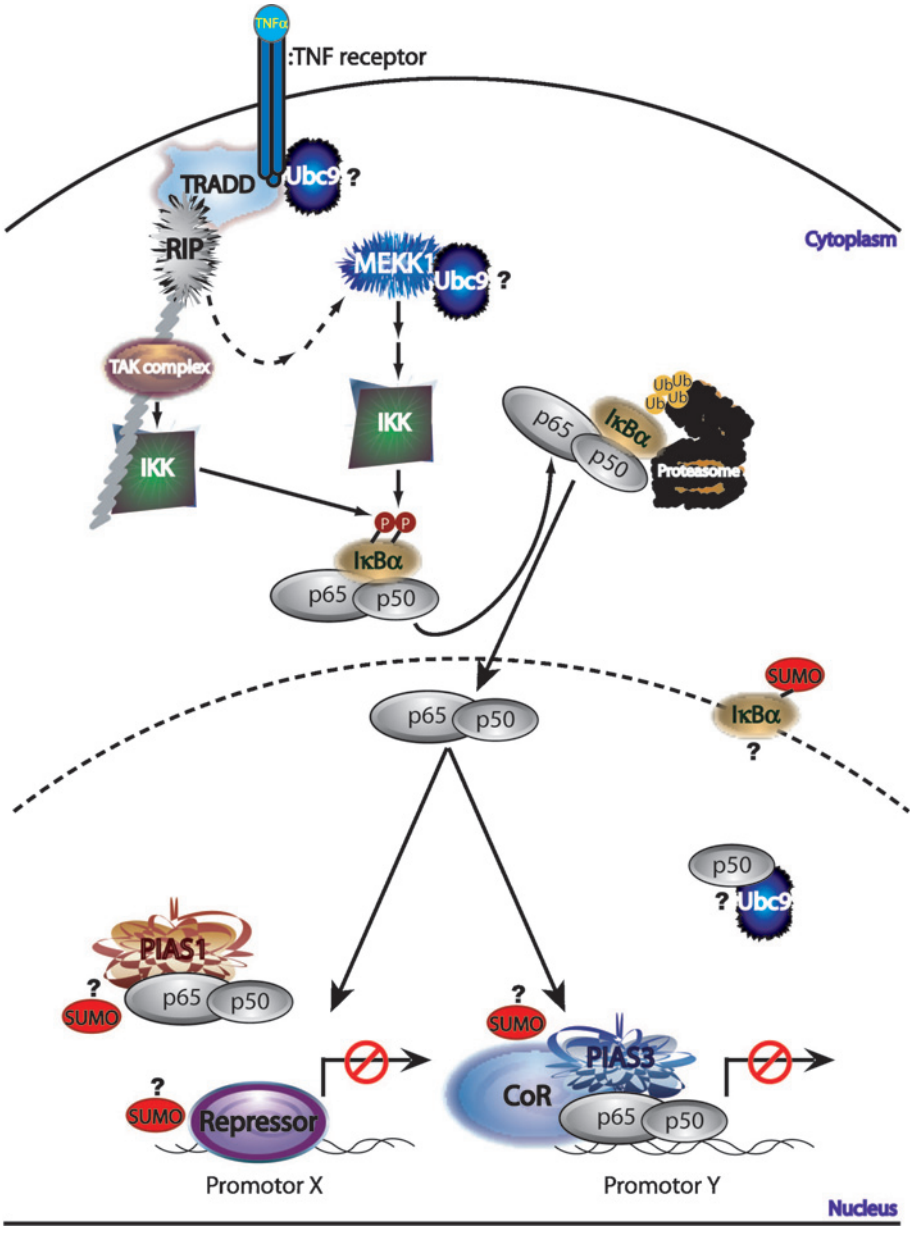

Figure 3. Model depicting possible roles for sumoylation and SUMO enzymes in TNFα-induced NF-κB activation. In response to TNFα treatment, TRADD and RIP are recruited to the death domain of the TNFR. In addition to the signaling pathway that involves non-canonical K63-linked polyubiquitination [125], MEKK1 could also participate in IKK activation. IKK then phosphorylates S32/S36 of IκBα, which promotes its ubiquitination and degradation by the 26S proteasome. Free NF-κB (p65/p50) translocates into the nucleus, where it can promote transcription of its target genes. Attenuation of NF-κB transcription through sumoylation or SUMO enzymes could be achieved in at least two ways: In the first mechanism, the SUMO E3 PIAS1 binds to NF-κB and inhibits its DNA-binding activity. PIAS1 may also be involved in the recruitment of a repressor that could prevent transcription of some NF-κB target genes. In the second mechanism, another SUMO E3, PIAS3, binds to NF-κB to inhibit its transactivation activity; this is most likely achieved through the recruitment of a corepressor complex. Whether or not sumoylation is involved in these two pathways is unclear. The physiological roles of the interactions of Ubc9 with p50, MEKK1 and TNFR1 are also currently undefined.

Perspectives and future studies

The critical roles of sumoylation and SUMO enzymes in NF-κB signaling continue to be elucidated using Drosophila and a variety of mammalian systems. SUMO and the SUMO enzymes seem to participate at the levels of membrane receptor, cytoplasmic signaling components, nuclear signaling components and nuclear transcriptional machinery to modulate NF-κB functions in a manner dependent on cell type and specific signals. Given the existence of many putative sumoylation sites in most NF-κB signaling proteins (see below), it is possible that the list of proteins that become modified by individual SUMO isoforms may expand in the near future. Even in situations in which specific substrates have been identified and their functions partially elucidated, such as IκBα and NEMO, there are many more issues that need to be resolved. For example, specific nuclear roles of SUMO-1-modified IκBα have yet to be defined. It is also uncertain whether IκBα is modified by endogenous SUMO-4. The potential involvement of a SUMO E3 (or E3s) and a protease (or proteases) also needs to be sorted out for this substrate. In the case of NEMO sumoylation, *in vitro* SUMO-1 modification by E1/E2 is extremely inefficient compared to IκBα sumoylation under the same conditions (Mabb, unpublished results). Even in the presence of PIASy, the sumoylation of NEMO is still an inefficient process. One explanation for this could be that the NEMO substrate

Table 2. Frequency of potential sumoylation motifs on proteins involved in NF-κB signalling.

Human	SUMO consensus Lysine sites	SUMO consensus Lysine sites with NDSM	Protein Accession Number
IKKα	109, 146, 479, 624	-	NP_001269
IKKβ	147, 238, 628, 704	704	AAC64675
IKKγ/NEMO	129, 277, 285, 325	129, 277, 325	Q9Y6K9
p65 (RelA)	37	-	AAA36408
RelB	33	33	NP_006500
p105/p50	86, 357, 491, 594	594	AAA36361
p100/p52	90, 298, 332	-	NP_002493
c-Rel	26, 112, 361	112	CAA52954
IkappaBα	21, 38	21, 38	AAA16489
IkappaBβ	187, 235	187	NP_002494
IkappaBε	-	-	NP_004547
IkappaBζ	209, 224	209	NP_113607
bcl-3	-	-	AAA51815
TNFR1	340	-	P19438
TRAF2	27, 313, 477	-	NP_066961
TRAF3	52, 107, 191, 429	191	NP_003291
TRAF5	100, 205, 287, 389, 417	205, 417	NP_001029082
TRAF6	67, 124, 142, 319, 453	142	NP_004611
βTrCP1	101	-	NP_378663
βTrCP2	65	-	NP_003930
MEK1	36, 205	36	NP_002746
JNK	-	-	NP_620637
TRADD	-	-	Q15628
TAB1	-	-	NP_006107
TAB2	329, 562	562	NP_055908
TAK1	547	547	NP_663304
A20	386	-	NP_006281
CYLD	201, 575, 665, 718	201, 575	NP_001035814
CARMA1	9, 177, 231, 237, 255, 726, 937, 1012	9, 177, 237, 255, 937	Q9BXL7
MALT1	262, 303, 702	262	Q9UDY8
Bcl10	110	-	O95999
TRIF	415	415	BAC44839
MYD88	161	-	AAC50954
TIRP/TRAM	36, 58	58	NP_067681
IRAK-1	-	-	NP_001020413
MEKK3	48, 65	48, 65	Q99759
Akt	64, 112, 182, 189, 276	112, 182, 276	NP_001014431
NIK	41, 54, 373, 734	41, 54, 373, 734	NP_003945
RSK1 (p90S6K)	119, 198	119, 198	NP_001006666
CKII	79	79	NP_001886
PIDD	-	-	AAG13461
RIP1	105, 140, 305, 565	140, 305	Q13546
ATM	24, 388, 640, 742, 1323, 1820, 1994, 2302, 2421, 2574, 2687, 2816	388, 640, 2421, 2816	AAB65827
ELKS	10, 195, 202, 439, 444, 472, 488, 579, 643, 706, 707, 712, 748, 885, 927, 962, 1078	195, 439, 444, 472, 579, 643, 706, 707, 1078	Q8IUD2
Proteins with SUMO sites	**37 out of 44**	**27 out of 44**	

Many proteins implicated in NF-κB signaling were analyzed for the existence of consensus SUMO modification motifs (ψKxE/D) using the SUMOsp program [118].
Consensus SUMO sites were then refined based on the NDSM (negatively charged amino acid-dependent sumoylation motif). The NDSM consists of acidic residues, namely aspartic and glutamic acid, flanking the SUMO consensus motif [119]. Out of 44 proteins, 37 contained consensus SUMO motifs and 27 contained SUMO consensus lysine sites with the NDSM.

employed is not in an appropriate state (lacking a modification possibly induced under cell stress conditions, in the wrong conformation or multimeric state, or a combination of these variables). This also highlights our lack of specific knowledge regarding the exact nature of "stress" and stress signaling induced by genotoxic agents to promote NEMO sumoylation. The identification of a SUMO protease and its role in regulating NEMO sumoylation also merits careful analyses. Such studies may also help to clarify how PIDD can act as a switch to promote NEMO sumoylation in the nucleus on one hand (*via* PIDD-C) and cell death through "PIDDosome" formation in the cytoplasm on the other (*via* PIDD-CC).

Although NEMO is currently the best characterized SUMO substrate in the NF-κB signaling system, there

are still many unanswered issues that pertain to this modification. One study indicated that nuclear NEMO could bind to CBP and prevent NF-κB-dependent transcription [114], while in another recent study, nuclear NEMO promoted the activity of the transcription factor hypoxia-inducible Factor 2α (HIF2α) [115]. Whether sumoylation of NEMO participates in modulation of these additional events remains to be investigated. Another relevant question is whether there are physiological signals that require NEMO sumoylation to cause NF-κB activation. In this context, it is also relevant to ask whether sumoylation of NEMO is required for NF-κB activation by genotoxic stimuli in most cell types under physiological settings. Sumoylation of NEMO has been described in a limited number of cell systems in response to different genotoxic stimuli [68, 82, 83, 86]. Thus, whether or not SUMO modification of NEMO and the putative SUMO modification sites (K277 and K309) are physiologically important *in vivo* remains undetermined. While mice deficient in PIASγ, the mouse homologue of human PIASy, have been generated and show no major observable developmental phenotype [50, 51], it is difficult to extract specific roles of NEMO sumoylation from this knockout mouse study due to the involvement of PIASy in regulation of different protein targets and potential compensation by other SUMO E3s. Thus, generation of knock-in mice for K270/302R-NEMO, the murine counterparts of human K277/309R, may be necessary to reveal the physiological role of NEMO sumoylation *in vivo*.

The variability seen with Ubc9 and its effects on NF-κB function currently appears confusing. The discrepancies amongst different model systems may involve both specific and pleiotropic effects of Ubc9 impinging directly on proteins in the NF-κB signaling pathway as well as indirectly on other proteins that are not directly involved in NF-κB signaling systems. Interpretation of results obtained with ectopic expression of Ubc9 and its mutants may be difficult, as this type of manipulation may exhibit global effects on cellular sumoylation processes, given that the levels of SUMO precursors, mature SUMO isoforms and sumoylated substrates seem to be tightly controlled in cells. Thus, additional careful analyses are required to reveal the functional significance of interactions between Ubc9 and substrates such as c-Rel, p50, and IκBα [58] and TNFR1 [112]. Similarly, whether PIAS1/3-mediated regulation of p65 involves sumoylation or is simply due to direct protein-protein interactions without the need for sumoylation remains unclear and requires additional studies; a comparison of catalytically active and inactive PIAS1/3 would be useful. There are examples, such as PIASy-mediated repression of the androgen receptor (AR) activity [116], in which PIAS family members can exert their negative transcriptional effects independent of their catalytic activity.

Although the knowledge of the roles of SUMO isoforms and SUMO enzymes in the regulation of NF-κB signaling and NF-κB transactivation is still incomplete, even less is known about SUMO proteases in NF-κB signaling and transactivation. As stated above, only six SUMO proteases have been identified [44]. In the Drosophila system, Drosophila Ulp1, similar to human SENP1, was shown to desumoylate Dorsal and inhibit Dorsal/Twist activation of a *Dorsal*-dependent reporter [103]. In HeLa cells, SENP1 inhibited TNFα- and p65-induced NF-κB luciferase reporter activity; however, this inhibition appeared to be independent of the catalytic activity of SENP1 [117]. For IκBα and NEMO, nothing is known about the specific involvement of SUMO proteases. Similar to the manipulation of SUMO isoforms and Ubc9, the levels and activities of individual SUMO proteases in cells likely impart a large-scale perturbation in the SUMO system. Thus, experimental setups and interpretation of results from SUMO protease manipulations also require careful consideration before defining a SUMO protease specifically involved at each of the signaling steps.

Since the knowledge of SUMO-modified substrates in the NF-κB signaling system is still very limited, we sought to identify potential SUMO substrates and putative sumoylation sites on known NF-κB signaling proteins by means of sequence analysis. Initially, we evaluated the presence of the consensus SUMO modification sites on known NF-κB signaling proteins through the use of a predictive consensus SUMO site program, SUMOsp (http://bioinformatics.lcd-ustc.org/sumosp/index.php), which predicts putative sumoylation sites within proteins based on previously identified SUMO motifs and the consensus ψKxD/E motif [118]. From this analysis, we identified potential SUMO sites in 37 out of the 44 NF-κB signaling proteins examined (Table 2). It was recently proposed that a potentially more accurate predictor of SUMO modification sites could be the identification of acidic residues flanking the SUMO consensus motif [119]. Using these parameters, we narrowed the number of potential SUMO-modified substrates in the NF-κB signaling system down to 27. It is unclear whether any of these sites are modified by SUMO under any physiological or pathological conditions *in vivo*, but it is noteworthy that so many of the NF-κB signaling proteins possess potential SUMO modification sites. However, this may also be a reflection of a generally high frequency of putative SUMO consensus sites, since when components of another signaling system

(the insulin receptor signaling pathway) were analyzed, a similarly large number of proteins were found to possess such a motif. As detection of sumoylated proteins in cells is difficult, a considerable effort may be needed to determine the whole repertoire of SUMO substrates in the NF-κB signaling system. Given the expanding physiological and pathological roles of both the SUMO system [32, 120, 121] and the NF-κB signaling pathways [122–124], the tie between these two fundamentally important cellular pathways is likely to expand substantially through future studies.

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